Isolation and characterization of novel actinobacteria and myxobacteria especially from marine habitats

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Posterbeiträge

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Abbreviations

°C	degree Celsius
16S rRNA	component of the 30S small subunit of a prokaryotic ribosome
$[M+H]^+$	protonated molecular irons
ASW	artificial sea water
cm	centi meter
DAD	diode array detector
dest.	destilled
DGC	"Diffusion Growth Chamber"
DNA	deoxyribonucleic acid
DNP	Dictionary of Natural Products
DSMZ	Deutsche Sammlung für Microorganismen und Zellkulturen
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-Spectrum-Betalaktamase
g	gram
GGDC	Genome-to-Genome Distance Calculator
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HRESIMS	high resolution electron spray ionization mass spectrometry
HZI	Helmholtz Zentrum für Infektionskrankheiten
ICBM	Institut für Chemie und Biologie des Meeres
JSRM	Jump Start Ready Mix
LC-MS	Liquid Chromatography – Mass Spectrometry
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
mg	mili gram
MIC	Minimal Inhibition Concentration
min	minute
m	meter
mm	milli meter
mL	mili liter
MMC	Marine Myxobacteria Cluster
MRSA	methicillin-resistant *Staphylococcus aureus*
m/z	mass-to-charge ratio
NADH	Nicotinamide-adenine-dinukleotide
NCBI	National Centre for Biotechnology Information
ng	nano gram
NMR	nuclear magnetic resonance
OD	optical density
PCR	polymerase chain reaction
pH	potential of hydrogen/ numeric scale used to specify the acidity or basicity of an aqueous solution
ROV	Remotely Operated Vehicle
RT / t_R	retention time
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SEM	emission scanning electron microscope

Soce	Sorangium cellulosum
VREF	vancomycin-resistant Enterococcus faecium
WS	Wadden Sea sediment
XAD	Scavenger (Amberlite XAD 16N from Rohm and Hass)
µg	micro gram
µm	micro meter
µL	micro liter

Abstract

The misconception that all bacterial infections could be cured with existing antibiotics resulted in a gap in antibiotics production, effectively ending the golden era of antibiotics. The subsequent loss of interest of big pharma-companies in developing new antibiotics as well as issues in the drug regulation, led to an increased number of multidrug resistant bacterial strains and a rapid rise of neglected diseases, over the last 25 years. Today, the identification of novel antibacterial scaffolds and their development into drugs has again become vital to human health and for that natural products have come into focus. Two very important sources to find biologically active natural products are the actinobacteria and myxobacteria. To find new strains as well as new secondary metabolites, this study deals with the isolation of these organisms especially from marine habitats.

This thesis is divided into 5 parts. The first part is dedicated to the isolation of marine myxobacteria from the "Marine Myxobacteria Cluster" (MMC). Therefore surface sediment was sampled from the Wadden Sea from northern Germany, the specific sequence for marine myxobacteria (MMC) was detected via specific PCR and diverse isolation approaches were started. Finally, 12 halotolerant *Myxococcus* strains were isolated which were closely related to terrestrial strains and do not harbour the MMC sequence. From these strains no new bioactive secondary metabolites could be detected.

Two parts focus on the taxonomical analysis as well as the screening for novel bioactive secondary metabolites from marine actinobacteria. These were isolated from living marine sponges or sediment from Guam and the rhizosphere sediment from mangrove plants from India. Taxonomically, strains ICN19 and 21 seemed to present a new species, however some further studies have to be done. For the screening of novel secondary metabolites a new multi resistant test panel could be established, consisting of Gram - positive and -negative bacteria as well as fungi including same multi resistant clinical relevant strains like methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VREF) and different antibiotic resistant *E .coli* strains. Rakicidins A, B and E as well as the anthraqiunones aloesaponarin II and 5-hydroxyaloesaponarin II were isolated. All five compounds exhibited bioactivity against Gram-positive bacteria, including the MRSA and VREF strains, which was never described before. Furthermore, 5-hydroxyaloesaponarin II was described to be produced by a bacterial wild type strain for the first time. Moreover the bioactive compound staurosporine was isolated from the marine *Streptomyces* strain ICN21.

Out of deep sea samples from the North Atlantic Ocean 4 marine *Streptomyces* strains were isolated. Using 16S rRNA PCR, MALDI-TOF, RiboPrinter® and DNA-DNA analyses as well as physiological and morphological approaches, the strain ASO4 wet was described as a new species.

In the last part of this thesis strain *Streptomyces davawensis* (roseoflavin producer) and the cinnabaramid producer JS360 were characterized by polyphasic taxonomy and identified as novel species of the genus *Streptomyces*

1. Introduction

Microorganisms are known to produce a plethora of secondary metabolites with a high structural variety and a wide spectrum of biological activity (Newman et al. 2003). Actinobacteria, especially *Streptomyces* strains, are one of the most importat sources for bioactive natural products with about two-thirds of the antibiotics from natural origin being produced by members out of this genus (Baltz 1998; Weber et al. 2003). However, during the last decades it became increasingly difficult to isolate new actinobacterial strains, producing novel structurally uncommon bioactive substances (Mohr 2016). Additionately, the number of antibiotic resistant strains increased (Cooper and Shlaes 2011), because basing on Davies (2006) "resistance develops within two or three years after the introduction of a new antibiotic treatment" (Mohr 2016). Hence, other microorganisms such as myxobacteria came into focus as producers of bioactive natural products (Clardy et al. 2006; Behal et al. 2003). Actino- as well as myxobacteria are known to live in soil (Dawid 2000; Madigan et al. 2009). This leads to the assumption that they release secondary metabolites to protect themselves as well as their food source against other bacteria and fungi. Unfortunately, it became harder to find new strains using big screening approaches (Balz 2006). Consequently, modern screening approaches have to focus on selected sources from special habitats for the isolation of new bacteria. Hence, this study is dedicated to the isolation of myxobacteria and actinobacteria from marine habitats, where both are known to be present (Dawid 2000; Lam 2006; Brinkhoff et al. 2011).

1.1. Natural products

1.1.1. Bioactive compounds /antibiotics

"Natural products" are products of natural origins. They include for example an entire organism (plant, animal or microorganism), a part of an organism (leaves or flowers of a plant or an isolated organ of an animal) parts or exudates of organisms or even secondary metabolites like alkaloids, glycosides sugars, steroids, etc. (Samuelsson 1999). Secondary metabolites are small molecules which are not essential for growth or development of the producing organisms but are by-products of their metabolism (Cannell 1998). However, most of the secondary metabolites are "end products of complex biosynthetic processes" (Habeck 2002) which were built by highly organized enzyme systems, called nonribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs), out of simple building blocks (Cane 1997; Staunton & Weissmann 2001; Rawlings 2001; Schwarzer et al. 2003; Schwarzer & Marahiel 2001). Microorganisms are producers of many different secondary

metabolites with a high structural variety and a wide spectrum of biological activity (Newman et al. 2003). The best-investigated microorganisms for the production of secondary metabolites are the actinobacteria (Challis et al. 2003). But also bacilli and pseudomonads are well known sources for secondary metabolites. However, the increasing resistance of pathogenic bacteria against known antibiotics (Walsh, 2003) and the rising recovery rate of already known antibiotics resulted in the discovery of new sources for antibiotic research (Fenical et al. 1999; Lam 2006; Donadio et al. 2010). Therefore, myxobacteria as well as cyanobacteria came more and more into the focus as bacterial producers (Clardy et al. 2006; Behal 2003). With more than 9000 strains, including all validly described type strains, the working group "Microbial Strain Collection" of the Helmholtz Centre for Infection Research (HZI, Braunschweig, Germany) harbour the largest collection of myxobacteria worldwide (Landwehr et al. 2016). Scientists of the HZI were able to isolate more than 500 new natural products from about 6000 different myxobacterial strains with at least 100 novel scaffolds (Gerth et al. 2003; Reichenbach 2001) exhibiting antifungal, antibiotic and cytotoxic biological activities (e.g. Irschik et al. 1983; Irschik et al. 1985a; Surup et al. 2014; Gerth et al. 1994; Geth et al. 1996). To screen for novel actinobacteria and actinobacterial secondary metabolites, the in-house strain collection of the HZI features more than 3000 actinobacterial strains and several collaborations with working groups all over the world. However, to detect the potential of the production of different secondary metabolites of a strain, a phenotypic analysis is not sufficient (Wenzel & Müller 2009). The biosynthetic potential cannot be fully utilised using standard fermentation conditions (Bode & Müller 2005) but with the help of variable cultivation conditions like changing media compositions, modifying the aeration (Bode et al. 2002) the transcription, translation and the enzyme activity profiles can be influenced (Wenzel & Müller 2009). The variation of the culture conditions and the resulting increase of the produced secondary metabolites was also shown in the "OSMAC (One Strain – Many Compounds) approach by Bode et al. (2002) who isolated more than 100 compounds which belong to 25 different structural classes from only six different microorganisms.

1.1.2. Need of new antibiotics

In contrast to the often described assumption that Alexander Fleming was the first scientist who discovered penicillin, misleadingly described as the first antibiotic compound from microbial origin in 1928, the antibiotic activity of mold has been described several times before (Mohr 2016). Particularly the scientific results of Ernest Duchesne which has been published in 1897 as "Contribution to the study of vital competition between microorganisms: antagonism between moulds and microbes" (Duchesne 1897) gave an interesting insight into the inhibitory growth of

bacteria effected by moulds. However, after the publication of Alexander Fleming in 1928, Florey and Chain worked on the structure elucidation of penicillin and with the help of Heatley the large scale production was possible so that finally in 1941 penicillin was introduced in therapy (Fleming 1929; Chain et al 1940; Mohr 2016). Nonetheless, Fleming revolutionized the screening method for bioactive compounds. He spread soil dilutions on diverse agar plates which were incubated with pathogens before and focused on the microorganisms causing a growth inhibition of the test strains (Mohr 2016). Finally, with the discovery of penicillin and the development of the new time and money reducing screening method by Fleming, the "golden age" of antibiotics (1950-1960) was initiated. This not only led to the discovery of new antibiotics but also created a new industry (Demain 2006). During this period of time, one half of the antibiotics which are in clinical use today, were generated. Unfortunately, with the intense use of antibiotics for human therapy and therapeutic as well as nontherapeutic use e.g. for animal farming, the number of resistant bacteria increased immensely (Davies 2006) and the need for new antibiotics never stopped. In the beginning of the 1990th antibiotic research changed into a combination of high throughput screening as well as chemistry- and computer-assisted design of small molecule ligands (Koehn et al. 2005). However, the commercial natural product research came back into focus because "the pipeline for new antibiotics was running dangerously low" (Walsh 2003). While the rate of newly discovered antibiotics decreased, the rate of re-isolations increased (Fenical et al. 1999; Lam 2006). Hence, the last few years have shown a significant increase in drug discovery costs leading to those screening approaches to be put down as scientific failures and even financial disasters. The time period of ten years was too long within costs and resistance development and newly discovered antibiotics were used as last-resort drugs which resulted in low sales for the new product. Moreover, "quality-of-life drugs" are more marketable and profitable (Davies 2006). As a result, more and more pharmaceutical companies stopped working on antibiotic research while the number of resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), the vancomycin-resistant *Enterococcus faecium* (VREF) or the fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP) increased (Cooper and Shlaes 2011).

Therefore, to discover new drugs, the focus has to be on new chemical classes or chemical classes which are known in research but new in the clinic. Strategies for this approach are the screening of microorganisms from unexplored environments and new taxa, the mining of the microbial genome, like the manipulation of the biosynthetic pathway, and the usage of new innovative assays (Donadio et al. 2010). This study is dedicated on the screening of actino- and myxobacteria, isolated with new isolation techniques from the marine environment (e.g. Wadden Sea, deep sea, sponges and mangroves). Furthermore, a new multi-resistant test panel was established for the screening of secondary metabolites.

1.1.3. Myxobacteria: Uncommon bacteria with a high potential as producers of natural products

Myxobacteria are gram-negative, rod shaped soil bacteria which live obligate aerobe and chemotroph. The first myxobacterium was described 1809 from the German botanist H.F. Link. However, because of its uncommon fungi-like life cycle, it was characterized as a fungus, "*Polyangium vitellinum* (Link 1809). More than 80 years later the American botanist R. Thaxter identified the myxobacteria as bacteria (Thaxter 1892). Today, myxobacteria belong to the δ-Proteobacteria and build the order Myxococcales. To date, this order consists of 55 species including 28 genera (Figure 1).

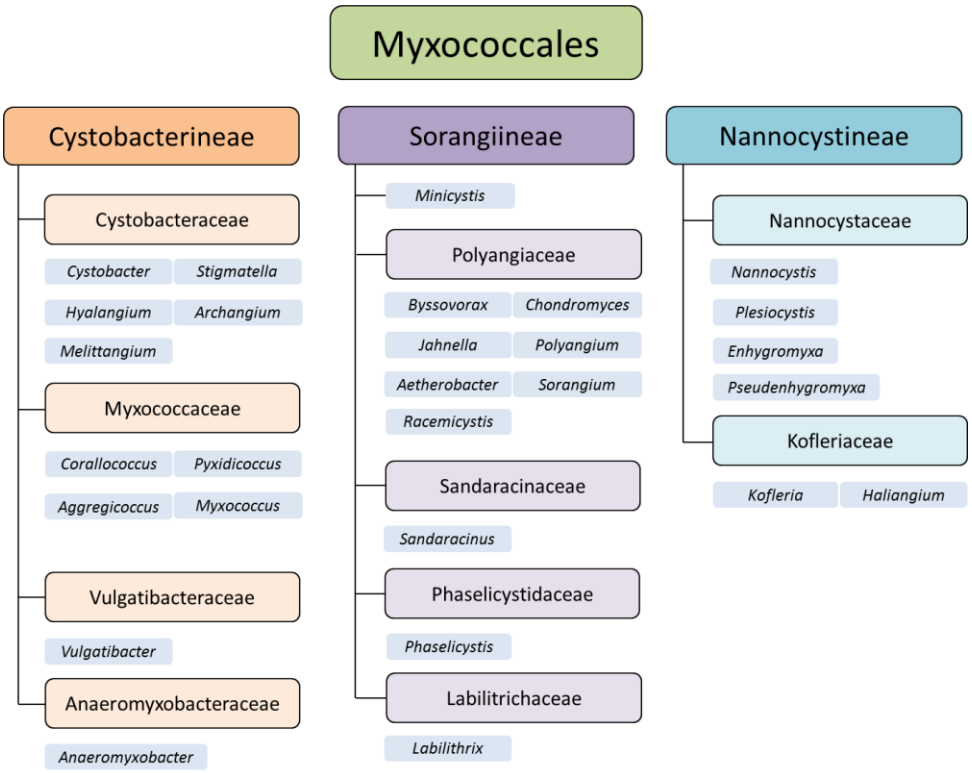


Figure 1: Current taxonomy of the order Myxococcales (Landwehr et al. 2016).

Myxobacteria differ to other bacteria in their uncommon behaviour during their lifecycle. Because of their ability to move in a defined direction on a thin slime film on hard substrate (Hodgkin et al. 1979), they are able to develop "social behaviour". At a lack of nutrients, they have the ability to move together to build fruiting bodies (Reichenbach 1986, Shimkets 1990) with up to 10^5 cells which differentiate into myxospores which are able to survive in a vegetative resting state till the environmental conditions changed. The shape of the fruiting bodies differs between the species from

tall and tree-like (*Stigmatella aurantica, Chondromyces crocatus*) to globular formations in *Angiococcus* and *Sorangium* species. Even the colour differs between yellow, orange or red until brown or even black (Reichenbach 1983, Garcia & Müller 2014a-e). To date, fruiting bodies as well as the different swarming formations (Figure 2) are highly important for the classification of the different species of myxobacteria (Reichenbach 2001) and remind sometimes more of eukaryotic myxomycetes or fungi than prokaryotic bacteria (Gerth et al. 2003).

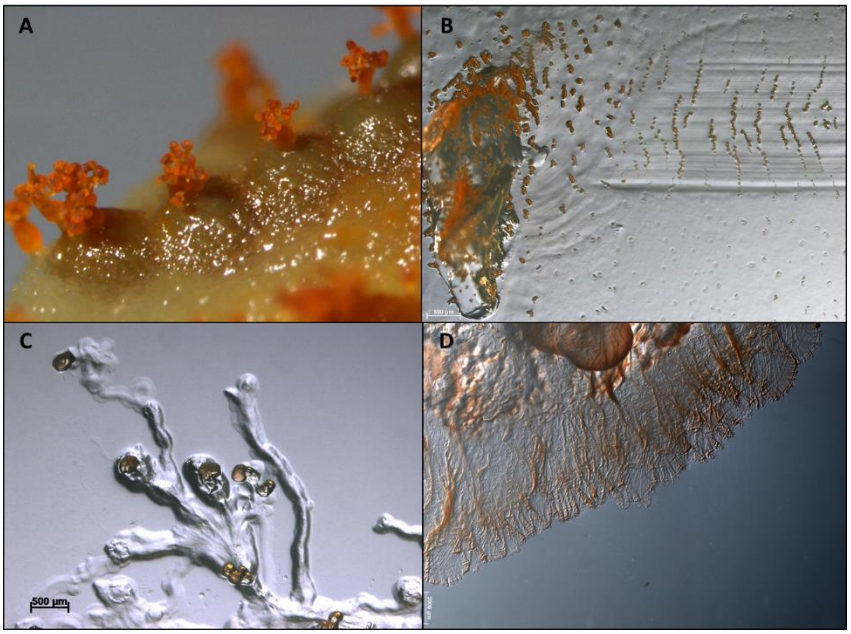


Figure 2: Fruiting bodies and swarming formations of different myxobacterial type strains. A: fruiting bodies of *Stigmatella aurantiaca* (Sga M15, DSM17044^T); fruiting bodies and swarming formation of B: *Corallococcus coralloides* (SBCo036, DSM2259^T); C: *Polyangium sorediatum* (Pls12, DSM14670^T); D: *Sorangium cellulosum* (Soce1871, DSM14627^T) (Microbial strain collection, HZI Braunschweig, Germany).

Moreover, because of their different nutrition requirements, myxobacteria are divided into two ecological groups, the predators and the cellulose decomposers. By excreting lytic exoenzymes, they are able to lyse other bacteria, yeasts or organic material like wood to take up proteins and nucleic acids (Reichenbach et al. 1988; Reichenbach et al. 2006).

Myxobacteria are also well known for their large genome with a very high amount of G-C interactions (Mandel et al. 1965). The model strain *Sorangium cellulosum* Soce56 has with a genome size of 13.04 Mbp and more than 10000 genes the largest known bacterial genome (Pradella et al. 2002). The large genome size was linked to the extraordinary and complex life cycle of the myxobacteria. For their special social behaviour, like swarming and the formation of fruiting bodies, they need a more complex genetic constitution than other bacteria (Wenzel & Müller 2009; Landwehr et al. 2016).

Myxobacteria are well known for the production of structurally diverse secondary metabolites, many of which show biological activity. For example, within the genome of Soce 56, 17 gen clusters were found which were involved in secondary metabolism (Schneiker et al. 2007). However, using the traditional screening methods only three metabolite families could be identified, yet (Weissman & Müller 2009; Irschik et al. 2007; Ambrosi et al. 1998; Kunze 1989, Jansen et al. 1997; Irschik et al. 1995). The great potential of secondary metabolite production has also been observed via genome screening of strain *Myxococcus xanthus* where 8.5% of the genome was identified to be involved in natural product biosynthesis (Goldmann et al. 2006; Weissmann & Müller 2009). In their natural habitat, most strains were isolated from soil, which is a highly competitive environment, myxobacteria have to defend their ecological niche. Cellulose degrading strains combat with cellulose degraders, e. g. fungi, and predatory strains lyse other bacteria decaying organic material (Gerth et al. 2003). Therefore it is not surprising that 29% of the myxobacterial compounds exhibit antibacterial and 54% antifungal properties (Weissman & Müller 2009). However to date, next to antibacterial and fungicidal effects, myxobacterial compounds act as antitumor drugs and show e.g. antiviral, insulin-sensitizing and immunoregulatory characteristics (Gerth et al. 1996; Reichenbach & Höfle 2008; Schreurs et al. 2009; Koutsoudakis et al. 2015; Berod et al. 2014; Corominas-Faja et al. 2014). The most important myxobacterial compounds with the highest pharmaceutical interest are epothilones A and B (Figure 3). They were isolated from a *Sorangium cellulosum* strain in 1996 and described as antifungal and cytotoxic agents (Gerth et al 1996). To date, modified versions of these compounds are in clinical trials against different types of cancer. As Ixempra® (ixabepilone) (Figure 3), developed in October 2007, a semi-synthetic epothilone B derivative is used for monotherapy of different stages of breast cancer (Reichenbach & Höfle 2008). However, during the last few years some very promissing compounds were described isolated from different types of myxobacteria showing diverse bioactivities. The antibiotics disciformycin A and B (Figure 3), isolated from *Pyxidicoccus fallax*, are biologically active against Gram-positive bacteria including MRSA strains (Surup et al. 2014). Corallopyronin A (Figure 3), isolated from *Corallococcus coralloides* (Irschik et al 1985b), was described to be a promising compound against filarial nematodes which cause lymphatic filariasis and onchocerciasis (Schäberle et al. 2014). Nannocystin A (Figure 3) was isolated from *Nannocystis* sp. (Hoffmann et al. 2015) and was described as inhibitor of the eukaryotic translation elongation factor 1α. This compound has an overlapping binding site with the anticancer compound didemnin B, whose derivates reached phase II in clinical trials, and therefore nannocystins “may serve as alternative starting point to develop new medicine” (Krastel et al. 2015). The macrolide chlorotonil A (Figure 3) which was isolated from a *Soranguim cellulosum* strain, was found to exhibit promising antimalarial activity and is proposed as a “lead structure for further development as an antimalarial chemotherapeutic” (Held et al. 2014). And finally, cystobactamides (Figure 3) which

show potent inhibitory effects against various *E. coli* strains and pathogenic Gram-negative strains like *A. baumannii* and *P. aeruginosa*. These compounds were isolated from the strain *Cystobacter velatus* (Baumann et al. 2016). In sum, myxobacteria are a promising source of new bioactive compounds.

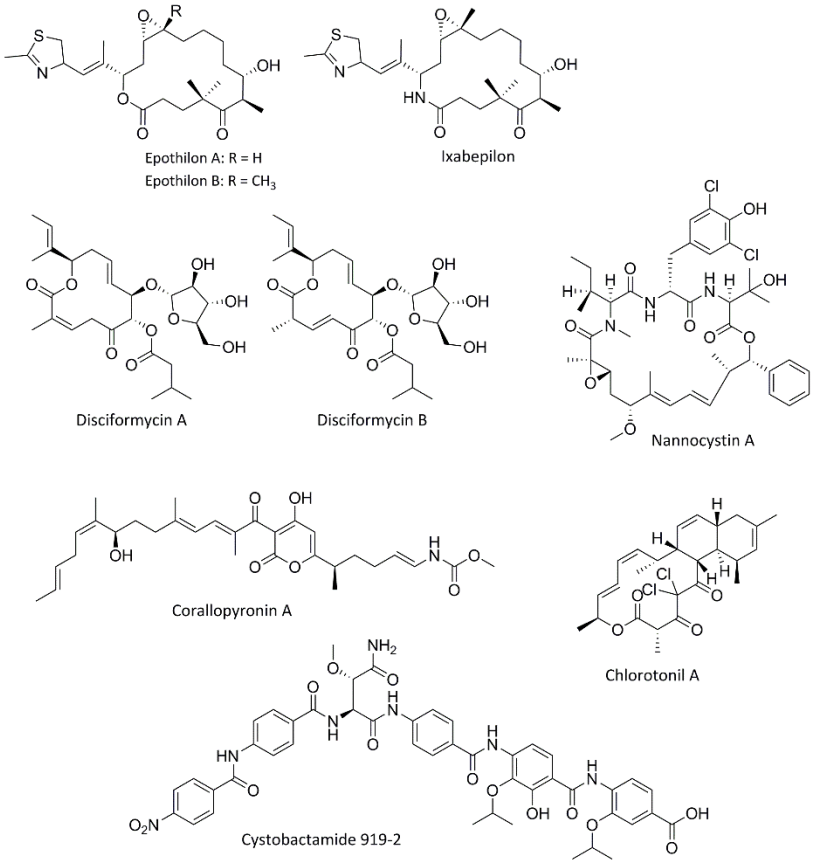


Figure 3: Chemical structures of interesting bioactive substances produced by terrestrial myxobacteria: epothilone A and B (and the semi-synthetic epothilone B derivative ixabepilone), disciformycin A and B, nannocystin A, corallopyronin A, chlorotonil A and the cystobactamides

1.1.4. Actinobacteria as producers of natural products

In contrast to the myxobacteria and *Pseudomonas* species, which are known as good producers for secondary metabolites, actinobacteria do not belong to the Proteobacteria. However, besides the Cyanobacteria which built a separate group in the domain bacteria, actinobacteria and the *Bacillus* species (Firmicutes) belong to the Gram-positive bacteria (Madigan et al. 2009). As mentioned above, these five bacterial groups are described to be the best producers of secondary metabolites. However, actinobacterial species, especially the filamentous *Streptomyces* strains, produce about three times more bioactive substances than the other producer strains in sum (Berdy 2005).

The phylum actinobacteria is „one of the major phyla in the domain *Bacteria*" (Goodfellow 2001a); Garrity & Holt 2001; Ludwig & Klenk 2005). Basing on Goodfellow (2001a), this phylum included five classes, 19 orders, 50 families and 221 genera. Thus, because of the rising discovery rate of members belonging to the phylum actinobacteria, these data are incomplete to date. The five classes are *Acidimicrobiia*, *Actinobacteria* (Stakebrandt et al. 1997), *Coriobacteriia*, *Rubrobacteria* and *Thermoleophilia*. However, because of the high amount of differences in morphology, physiology and G-C content, which differs between 50% and 70% within the diverse genera, this study focuses on the class *Actinobacteria* for further description. This class is divided into 15 orders, 43 families and 203 genera. They are Gram-positive bacteria which diverse morphological appearance differing between cocci, short rods, irregular rods, rods and cocci, mycelia which fragments into coccoid and rod like elements as well as the development of branched substrate hyphae which bears spores or spore vesicle or the formation of stable branched mycelium with aerial hyphes that differentiate into short or long spore chains or spore vesicles, respectively (Goodfellow et al. 2001b). Actinobacteria are described to be common soil inhabitants (Berdy 2005), however they are also widely distributed in the marine environments and extreme habitats like deep sea sediments (Pathom-aree et al. 2006), and in soil of hyper arid deserts (Okoro et al. 2009). In soils they are amongst others responsible for the turnover of organic material and recalcitrant of molecules (Goodfellow and Simpson 1983; Goodfellow and Williams 1983). But some of them are also found as animal or plant pathogens, plant commensals, and nitrogen- forming symbionts or as inhabitants of the gastrointestinal tract (Goodfellow et al. 2001b).

Moreover, the class Actinobacteria is considered as the richest source for bioactive natural products (Goodfellow et al. 2001b; Berdy 2005; Newman and Cragg 2007; Olano et al. 2009), because 45% of the bioactive substances in clinical use today were produced by filamentous bacteria of which 80% are products of members of the genus *Streptomyces* (Berdy 2005; Newman and Cragg 2007; Olano et al. 2009). The discovery of penicillin and the World War II led to the "intensive search for new synthetic and natural antibiotics around the 1940th" and the focus of this search lay on the screening of "microorganisms from the environment for antimicrobial activity" (Mohr 2016). The biochemist Waksman and his students screened soil bacteria basing on the new screening method described by Fleming and detected bacteria from the genus *Streptomyces* as "highly promissing candidates for the production of new antibiotics" (Mohr 2016). The first antibiotic compound described from an actinobacterium was the substance actinomycin in 1940. It was isolated from *Streptomyces antibioticus* subsp. *antibioticus* and showed biological activity against a broad range of bacteria and even against the tuberculosis strain *Mycobacterium tuberculosis*. However, it was too toxic against

human cells for the usage as a therapeutic agent (Waksman & Woodruff 1940; Waksman & Woodruff 1941). But actinomycin D was found to have a significant anticancer activity and therefore it is still in use in antitumor therapy (Mohr 2016). Only four years later the first aminoglycoside antibiotic had been discovered. The potent broad-spectrum antibiotic streptomycin was isolated from *Streptomyces anulatus* subsp. *griseus* in 1944 by Schatz et al. and co-discovered by Waksman and colleagues who were awarded the Nobel Prize in 1952 (Mohr 2016). Streptomycin was the first described antibiotic with an activity against tuberculosis which was used in therapy (1946) but also showed biological effects against meningitis (Hamdy 2006; Mohr 2016). Also the other aminoglycosides like kanamycin and gentamycin were described as "milestones" because of their activity against infectious diseases caused by Gram-negative bacteria (Mingeot-Leclercq et al 1999; Shaw et al. 1993; Mohr 2016). This was the beginning of the golden age of antibiotics largely evoked by actinobacterial research. However, most of the antibiotics discovered in this time, are still in clinical use today (Mahajan 2012). The most famous antibiotics from the "golden age" are the before mentioned aminoglycoside streptomycin (Schatz et al. 1944) as well as the broad-spectrum aminoglycoside neomycin (Waksman & Lechevalier 1949) which was isolated from *Streptomyces fradiae* and is active against streptomycin-resistant bacteria, kanamycin, produced by *Streptomyces kanamyceticus* (Takeuchi et al. 1957) and mainly used as therapeutic agent for eye infections and as reserve antibiotic (Mohr 2016) and gentamicin which was isolated from *Micromonospora purpurea* (laboratories of the Schering Corporation, Bloomington, New Jersy 1967 ; no authors listed) and showed a high antibiotic activity against almost all Enterobacteria, especially against the Gram-positive Staphylococci. Unfortunately, because of its strong side effects it is only used as an emergency antibiotic (Mohr 2016). Further examples for interesting antibiotics produced by actinobacteria are the broad-spectrum antibiotic chloramphenicol, produced by *Streptomyces venezuelae* (Ehrlich et al. 1947; Ehrlich et al. 1948; Smith et al. 1947; Gottlieb et al. 1948), the broad-spectrum antibiotics tetracyclines aureomycin (chlortetracycine) (Duggar 1948), terramycin (Finlay et al. 1950) and the structurally atypical chelocardin (Oliver et al. 1962; Mitscher et al. 1970; Oliva et al. 1992), the broad-spectrum macrolide antibiotic erythromycin (McGuire et al. 1952) which is to date the standard antibiotic against infections of the respiratory tract (Mohr 2016) and glycopeptides vancomycin (McCormick 1955) and teicoplanin (Parenti et al. 1978) which are exclusively active against Gram-positive bacteria and serve as reserve antibiotics because of their activity against bacteria showing a resistance to other antibiotics (Kahne et al. 2005; Mohr 2016), to name just a few. Furthermore, there are also some "old" antibiotics which came back into the focus because of their interesting bioactivity. A good example is the bioactive substance griselimycin (Terlain & Thomas 1971) whose activity was first described in 1973 in a patent of Mancy, Ninet and Preud, but the name was never validated and the describtion was incomplete. However, because of its specific activity

against mycolic acid-containing Gram-positive bacteria it came back into the focus in the 21st century. During the last years, the biosynthesis gene cluster was identified and characterized (Broenstrup et al. 2012) and a taxonomic analysis of the producer strain *Streptomyces caelicus* was published (Wink et al. 2016). Today some new optimized derivates of griselimycin are known which are active against tuberculosis, both in *vivo* and in *vitro* (Kling et al. 2015). However, the difficulties to isolate new actinobacteria during the last years with the common isolation methods (Fenical 1999) and the connected challenge to find new carbon skeletons and with this new bioactive compounds in *Streptomyces*, led to the isolation of new actinobacteria from unexplored habitats (Lam 2006). The biggest almost unexplored habitat is the ocean, which is described to be a promising new source for the discovery of new bioactive compounds (Fenical & Jensen 2006).

1.2. Marine environment

70% of the world's surface is covered with water and "with a microbial abundance of 10^6 per mL in sea water and 10^9 per mL in ocean bottom, oceans are the world's biggest environment" (Fenical & Jensen 2006; Landwehr et al. 2016)

1.2.1. Marine myxobacteria

1.2.1.1. Are there "real" marine myxobacteria?

Like mentioned before, myxobacteria have been described for a long time as soil bacteria which are common in many terrestrial environments (Dawid 2000; Reichenbach 1999). However, during the last years, more and more myxobacteria were isolated from limnic or even marine habitats. Because of their close relationship to their terrestrial relatives, Reichenbach (1999) suggested the limnic as well as the marine isolates to be no indigenous organisms but washed in from the terrestrial surroundings. Furthermore, most of the strains isolated from marine habitat were able to grow on rather low salinities (Li et al. 2002). Contrary to these findings, in 1998 Iizuka et al. isolated the first myxobacteria from marine samples from the coast of Japan which were adapted to marine habitats. Those strains need sodium chloride for growth and form fruiting bodies on media containing NaCl in concentrations from 2 to 3%, which are comparable to sea water. These bacteria were classified as new myxobacterial genera and described as *Haliangium sp*. (*H. ochroceum* and *H. tepidum*) and *Plesiocystis sp.* (*P. parcifica*), a few years later (Fodou et al. 2002: Iizuka et al. 2003a). Furthermore, Iizaka et al. (2003b) also described the new genus *Enhygromyxa* (*E. salina*), which was also well adapted to live in the marine environment. However, despite the new genera of the isolated marine

myxobacteria all of them belong to the genus *Nannocystis*. Basing on Zhang et al. (2005) the myxobacteria described to date could be divided into nonhalotolerant, halotolerant and halophile strains. Due to their ability to grow with and without NaCl as well as the phylogenetic relationship to the terrestrial isolates, members from the three myxobacterial genera *Haliangium*, *Plesiocystis* and *Enhygromyxa* were classified as halotolerant myxobacteria "which might form a different evolutionary group that is indigenous in the ocean" (Zhang et al. 2005). However, during molecular analysis of different marine sediment samples Jiang et al. (2010) established myxobacteria-enriched libraries of 16S rRNA gene sequences and found similar sequences which were closely related to terrestrial myxobacteria strains but simultaneously they were phylogenetically separate with a distance of about 10%. In the phylogenetic tree, clones harbouring this sequence clustered together and were named as the "Marine Myxobacteria Cluster (MMC)" (Brinkhoff et al. 2012). This cluster included, besides the generated clone sequences from the Wadden Sea samples, various clone sequences and sequences from uncultured bacteria, isolated from divers marine habitats from all over the world.

1.2.1.2. Bioactive substances produced by marine myxobacteria

To date only members of the halophile genera *Haliangium*, *Plesiocystis* and *Enhygromyxa* are described as marine myxobacteria. Therefore, the antifungal compound haliangicin (Figure 4) was the first bioactive compound isolated from a myxobacterium from marine origin. Haliangicin was isolated from *Haliangium luteum* and showed high activities against filamentous fungi but no antibacterial activity. Furthermore, it belongs to the class of compounds with a ß-methoxyacrylate moiety and is a respiratory inhibitor. It inhibits the electron transport within complex III of the respiratory chain (Fudou et al. 2001a, b). Moreover, twelve years after the discovery of haliangicin a second metabolite was isolated from an obligate marine myxobacterium. Salimabromide (Figure 4) was isolated from an *Enhygromyxa salina* strain and showed moderate antibiotic activity against the Gram-negative bacterium *Anthrobacter cristallopoedes* (Felder et al. 2013). With its unusual structure, salimabromide emphasized the phylogenetical distance of the producer strain with its terrestrial relatives. Further bioactive metabolites like the miuraenamides were only described from slightly halophilic myxobacteria like "*Paraliomyxa miuraensis*". The compounds miuraenamide A-F (Figure 4) were more or less active against the phytopathogen *Phytophthora capsici* (Ojika et al. 2008) However, the derivates miuraenamides A and B were described to have inhibitory effects against the NADH oxidase (Iizaka et al. 2006).

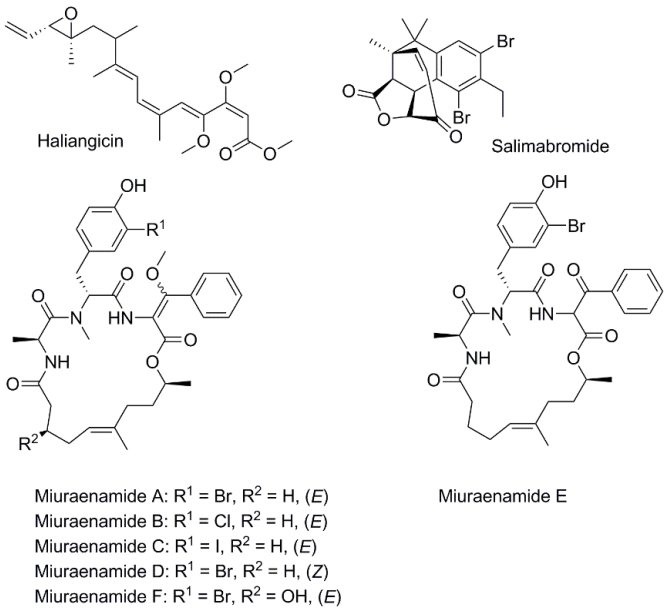


Figure 4: Chemical structures of bioactive natural secondary metabolites produced by marine / halophilic myxobacteria: haliangicin, salimabromide and miuraenamides A-F.

1.2.2. Marine actinobacteria

1.2.2.1. Are there "real" marine actinobacteria?

Actinobacteria are like myxobacteria common soil bacteria. Because of this it was a long time not clear, whether "real" marine actinobacteria did exist. Most people suggested that because of the high amount of re-isolation of known terrestrial strains from marine samples, "marine" actinobacteria are the result of wash-in of terrestrial spores into the marine habitat (Goodfellow & Haynes 1984). Another reason for this assumption could be that, because of the difficulty of sampling within the marine habitat, most of the samples have been taken close to the coast (Fenical & Jensen 2006). However, the strain *Rhodococcus marinonascence* was the first isolate which was described as marine actinobacterium (Helmke & Weyland 1984). A few years later, the first actinobacteria described as strains with "specific marine adaptations" (Jensen et al. 1991) or strains which were "metabolically active in marine sediments" (Moran et al. 1995) were published. The real breakthrough of marine actinobacteria came with the discovery of the first obligate marine genus *Salinispora* spp. in 1991 (Jensen et al. 1991). Members from this genus require sea water for growth. However, even about 10 years later, Mincer et al. described in 2005 the genus "*Salinospora*" (grammatically incorrect; corrected to *Salinispora*) by analysing the relationship of this genus with its terrestrial relatives and detected the widespread population of this genus in ocean sediments via DNA sequence-based methods (Mincer at al. 2002, 2005). Furthermore, based on the active growth in some sediment samples, Mincer demonstrated the ability of *Salinispora* strains to be metabolically active in their marine habitat (Mincer et al. 2005). Some *Salinispora* strains were also isolated from

the marine sponge *Pseudoceratina clavata* (Kim et al. 2005) collected from the Great Barrier Reef. For the formal description of this genus, the type strains *Salinispora tropica* and *Salinispora arenicola* were published in 2005 (Maldonado et al. 2005) and in 2014, the third type strain *Salinispora parcifica* was described (Ahmed et al. 2014). These findings encouraged many scientists to further work on marine actinobacteria. Starch and Bull (2005) found 1300 different actinobacterial operational taxonomical units in deep sea sediments which indicate the presence and great opportunity to isolate novel species and genera. According to this finding, Fenical and Jensen (2006) were able to isolate some marine strains which belong to six different actinobacterial families and seemed to represent new taxa like *Mariniphilus* and the above described *Salinispora.* To date, it is known, that actinobacteria are present in diverse marine habitats: from the sea surface layer over the water column down to the sea surface with its micro- and macro-fauna and –flora to the sea subfloor and the deep biosphere (Ward and Bora 2006).

1.2.2.2. Bioactive substances produced by marine actinobacteria

In addition to the taxonomic findings of the marine actinobacteria, it turned out that these strains are also “excellent producers of secondary metabolites” (Fenical and Jensen 2006). They produce a great spectrum of metabolites which can be used for diverse biotechnological applications like the production of melanins, single cell proteins and probiotics in aquaculture as well as enzymes and enzyme inhibitors. However, this study focuses on the production of bioactive metabolites like antibiotics, cytotoxic agents and fungicides (Manivasagan et al. 2013).

Most of the bioactive compounds described from marine actinobacteria are produced by strains from the genus *Streptomyces*. For example the antibacterial frigocyclinone from *Streptomyces griseus* (Brutner et al. 2005), the daryamides from *Streptomyces* sp., which have antifungal and anticancer activities and the antitumor agents chromomycins B, A2 and A3 produced by *Streptomyces coelicolor* (Lu et al. 2012). However, there are a few interesting compounds described, which were produced by members from the only obligate marine actinobacteria genera *Salinispora* and *Marinospora* (Manivasagan et al. 2013). Salinosporamide A (Figure 5) is the most promising bioactive compound. Becides salinosporamide B (Figure 5), it is produced by *Salinispora tropica* and is an orally active proteasome inhibitor which induces apoptosis in multiple myeloma cells. Its mode of action differs from the mechanism of the commercial proteasome inhibitor anticancer drug bortezomib (Chauhan et al. 2005; Manivagasan et al. 2013). As the first compound isolated from an obligate marine actinobacterium, salinosporamide A (also known as NPI-0052) entered the clinical studies in multiple phase I trials for solid tumours, lymphoma and multiple myeloma

(http://www.nereuspharm.com/NPI-0052.shtml). The structure of salinosporamide B differs only by the lack of a chlorine atom, which decreases the activity by a factor of 500 (Manivasagan et al. 2013). Both compounds are unusual bicyclic γ-lactams. However, the cinnabaramides, which are produced by the terrestrial *Streptomyces* strain JS360 are chemically closely related to the salinosporamide A. These compounds also act as selective proteasome inhibitors but show weaker cytotoxic bioactivities (Stadler et al. 2004; Stadler et al. 2007; Rachid et al. 2011). Except salinosporamide A and B, *Salinispora tropica* was also described as producer strain for sporolide A, arenicolide A, cyanosporaside A and salinipyrone A (Figure 5) (Jensen et al. 2007; Manivasagan et al. 2013). The bioactive compounds salinipyrones A and B (Figure 5), first described to be produced by *Salinispora parcifica* (Oh et al. 2008), cause a moderate inhibition of the interleukin-5 production without cell cytotoxicity on human cells. However, they did not show any antibiotic effect on human pathogens (Manivasagan et al. 2013). Marinomycin A (Figure 5) is the only compound described to be produced by a strain from the obligate marine genus *Marinophilus*. The polyene macrolide displayed a high toxicity on tumour cells and had in addition an antibiotic effect on vancomycin-resistant *Enterococcus faecium* and methicillin–resistant *Staphylococcus aureus* strains (MIC values of 0.1-0.6µM) (Kwon et al. 2006).

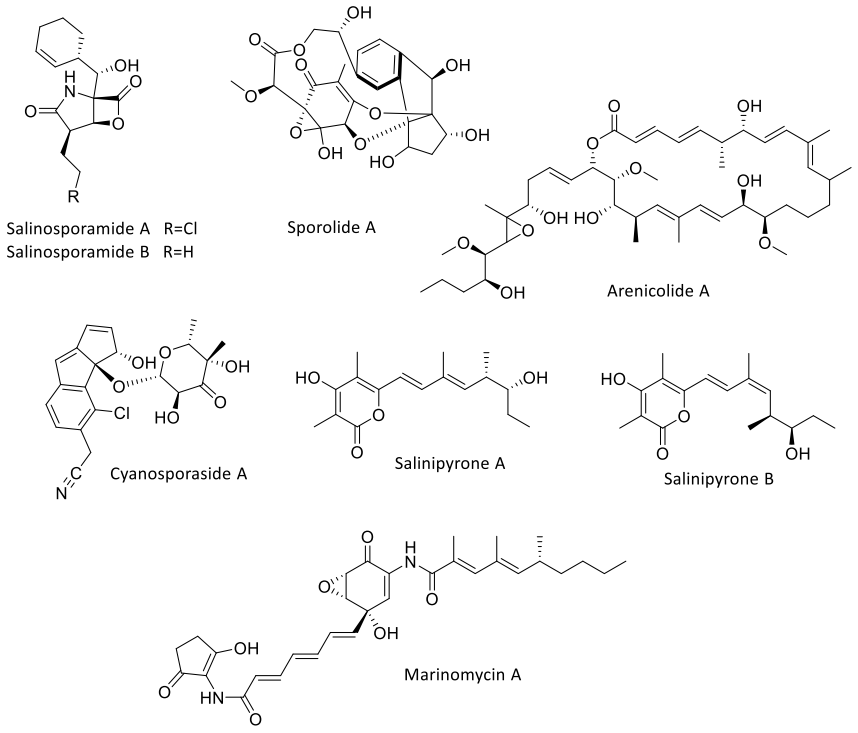


Figure 5: Examples of new bioactive compounds and their corresponding structures produced by novel genera of marine actinobacteria: salinosporamide A and B, sporolide A, arenicolide A, cyanosporaside A, salinipyrone A and B, marinomycin A.

1.3. Working projects and previous work

1.3.1. Part 1: Isolation of marine myxobacteria from the sediment of the Wadden Sea: search for the MMC sequence and screening for new bioactive compounds

In 2010, Jiang et al. detected myxobacterial 16S rRNA sequences in marine sediment samples which were closely related to terrestrial myxobacteria strains on the one hand but simultaneously clustered phylogenetically in separate clades with a distance of more than 10% on the other hand. Based on this study, Brinkhoff et al. (2012) developed specific primer (MMC655f and MMC841r) to detect members of this clade which was named the "Marine Myxobacteria Cluster" or MMC. In their study they showed the worldwide distribution of the MMC sequence in marine sediment samples almost all over the world to a water depth of 4300 m. It was detected in sediments from various climate regions like the Mediterranean Sea, the Atlantic, the Pacific, the Indian Ocean and from the North Sea where the MMC was found to make up 13% of the total bacteria 16S rRNA genes in the surface sediments. In addition to the molecular approaches like the construction of fosmid libraries und the phylogenetic analysis of the MMC sequences, they also tried to isolate marine myxobacteria harbouring the MMC sequence from marine sediments from the coast of Neuharlingersiel (North Sea, Germany). Different enrichment approaches were used but at last two *Myxococcus* sp. strains which did not belong to the MMC were almost cultivated (Brinkhoff et al. 2012).

1.3.2. Part 2: Actinobacteria isolated from marine sponges and sediments from Guam: Taxonomic characterization and screening for new bioactive compounds

The working group of Prof Dr. Peter Schupp from the University of Oldenburg works with marine sponges and the microorganisms living within these sponges. Commonly used methods for the isolation of microorganisms living within sponges are stamping techniques or even plating of different dilutions of crushed sponges (e.g. Mantalvo et al. 2005; Abdelmohsen et al. 2010; Selvin et al. 2004; Jiang et al. 2007). For all of this approaches the living sponge had to be harvested. However, Steinert et al. (2014) constructed a so called "Diffusion Growth Chamber" (DGC). This chamber is made out of two combined centrifuge micro filter and includes inoculation media. The filter membranes of both sides allow the diffusion of nutrients and waste products in and out of the chamber. This DGC can be inserted into the sponge and retaken after a certain incubation time (Figure 6). This isolation method keeps the sponges alive.

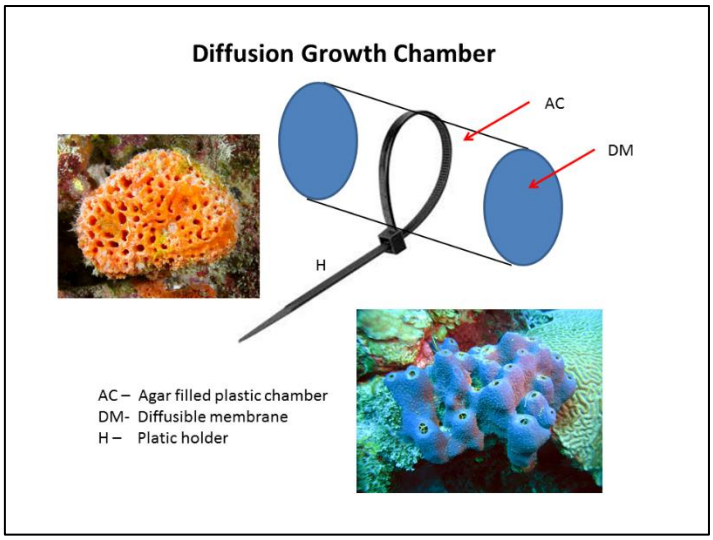


Figure 6: Diffusion growth chamber (DGC) for in vivo cultivation of sponge-associated bacteria. Build-up of DGC out of two combined centrifuge micro filter sections with diffusible membranes (DM), inoculated with different types of media within the chamber (AC) and the plastic holder (H) for fixing within the sponge.

With the help of the DGC, members of the working group of Prof. Dr. Schupp isolated diverse bacterial strains from the sponge *Rhabdastrella globostellata* at the coast of Guam as well as from marine sediments from the Blue Hole in Guam. For taxonomic characterization as well as for the screening for new bioactive compounds, 10 actinobacterial strains were analyzed during this study.

1.3.3. Part 3: Actinobacteria isolated from marine and rhizosphere sediment from mangroves in India: Taxonomic characterization and screening for new bioactive compounds

The working group of Dr. S. G. Prakash Vincent from the International Centre for Nanobiotechnology (ICN) of the Manonmaniam Sundaranar (MS) University in India isolated actinobacteria from rhizosphere sediment of mangrove plants *Avicinnia officinalis*, *Acrostichum aureum* and *Rhizophora mucronata* as well as from marine sediment. The samples were taken at a fish farm by the side of the Centre for Marine Science and Technology from the MS University (Figure 7A) and the Manakkudy Mangrove environment at the southwest coast of Tamil Nadu (India) (Figure 7B).

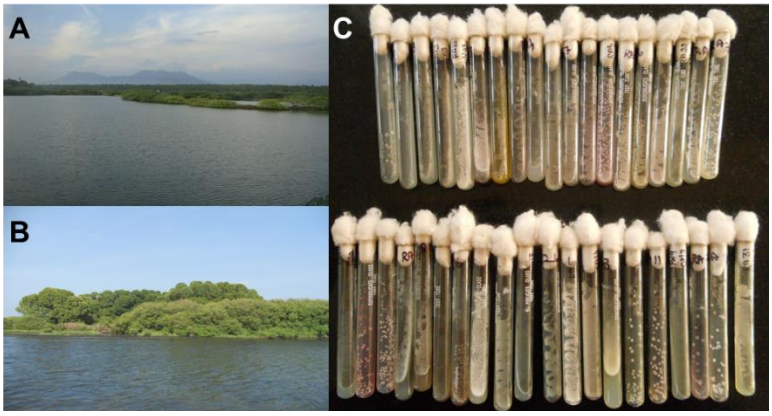


Figure 7: Sampling sites and isolated ICN strains of the Manonmaniam Sundaranar (MS) University in India. A: fish farm by the side of the Centre for Marine Science and Technology from the MS University; B: Manakkudy Mangrove environment at the southwest coast of Tamil Nadu (India); C: Agar-shakes of isolated ICN strains.

For the taxonomic classification and the screening for secondary metabolites, 10 strains which belong to the actinobacteria were analyzed in this study.

1.3.4. Part 4: Actinobacteria from deep sea samples from the North Atlantic Ocean: Isolation, taxonomic characterization and screening for new bioactive compounds

In December 2014 the research vessel "Sonne" cruised through the North Sea and parts of the North Atlantic Ocean. Prof. Dr. Peter Schupp from the University of Oldenburg sampled two sponges and one soft-coral with the help of a ROV as well as sediment from 1092 m depth. Some previously prepared plates were directly inoculated and send to our working group to the HZI in Braunschweig for the isolation of new marine actinobacteria.

1.3.5. Part 5: Polyphasic description of *Streptomyces* strains JS360 and *S. davawensis*

During a screening program for new antibiotics, strain *Streptomyces davawensis* (DSM 101723^T) was isolated and detected to produce a red compound which showed biological activity against Gram-positive bacteria (Otani et al. 1974; Shinobu 1974). Because of its structural similarity to riboflavin (vitamin B_2) and the red colour of the compound it was named roseoflavin. This compound is the only known natural riboflavin analogue with antibiotic function and therefore presents a new class of antibiotics with new target structures. It is taken up by many bacteria via riboflavin transporters (Pedrolli et al. 2013; Grill et al. 2008; Hemberger et al. 2011; Vogl et al. 2007). During the search of related bacteria producing roseoflavin, Jankowitsch et al. (2011) detected strain *S. cinnabarinus* as close relative via 16S rRNA sequence which also used the same pathway as *S. davawensis* for the roseoflavin biosynthesis. In parallel we worked with the cinnabaramide producer JS360 which was

also described to be a member of *S. cinnabarinus*. However, instead of roseoflavin, this strain produced the cinnabaramides A-G, wich were described as proteasome inhibitors and are interesting substances for the treatment of allergies, asthma and cancer. The cinnabaramides are structurally close related to the marine compound salinosporamide A (Stadler et al. 2004; Stadler et al. 2007, Kyle et al. 2004).

In this study *S. davawensis*, *S. cinnabarinus* and strain JS360 were analyzed using a polyphasic strain description approach to clarify the phylogenetic relationship between all three strains.

1.5 Aim of the study

The aim of this study is divided into five different parts.

- Isolation of new marine myxobacteria from sediment from the Wadden Sea and screening for new bioactive metabolites (collaboration with the working group of Prof. Dr. Simon, ICBM (University of Oldenburg))
- Characterization of new actinobacteria isolated from marine sponges and sediments from Guam and screening for new bioactive compounds (collaboration with the working group of Prof. Dr. Schupp, ICBM (University of Oldenburg))
- Characterization of new actinobacterial strains isolated from marine and rhizosphere sediments from mangroves from India and screening for new bioactive compounds (collaboration with the working group of Dr. Vincent, Centre for Marine Science and Technology Mananmanian Sundarana University of Rajakamangalam, India)
- Isolation and characterization of new marine actinobacteria from deep sea samples from the North Atlantic Ocean and screening for new bioactive compounds (collaboration with the working group of Prof. Dr. Schupp, ICBM (University of Oldenburg))
- Characterization and phylogenic arrangement of *S. davawensis* and the cinnabaramide producer JS360 (*S. cinnabaragriseus*) (collaboration with the working group of Prof. Dr. Mack, University of Mannheim)

2. Material and Methods

2.1. Material

2.1.1. Media

All media used in this study are listed in table 1. They were freshly prepared after the recipies described below with destilled water and the pH was adjusted with 1 M NaOH or rather HCl solution. Afterwards the media were sterilised for 20 min at 121 °C.

Table 1: Media and ingredients used in this study.

Media	Ingredients/manufacturer
Basal medium (pH 7.0)	1% casein peptone 0.5% yeast extract 2% Bacto Agar (1.5% Bacto Agar) dest. water
CY-medium (pH 7.2)	0.3% casiston 0.1% yeast extract 0.1% $CaCl_2$ 50 mM HEPES (11.8 g/L) dest. water
CY / H-medium	50% CY- / 50% H-medium
DULBCCO´s modified EAGLE´s medium	DMEM, Bio Whittaker, Walkersville, MD
E-medium (pH 7.4)	0.4% skimmed milk 0.4% soy flour 1.0% starch 0.1% $MgSO_4$ 50 mM HEPES (11.9 g/L) 8 mg/L Fe-EDTA 0.5% glycerine dest. water
Eluent A2	50mL acetonotrile 385mg ammonium acetate 40 µL acetic acid 950 mL H_2O

Eluent B2	950 mL acetonitrile 385 mg ammonium acetate 40 µL acetic acid 50 mL H_2O
GYM-medium (pH 7.2)	0.4% glucose 0.4% yeast extract 1% malt extract 0.2% $CaCO_3$ dest. water
GYM-ASW (pH 7.2)	0.4% glucose 0.4% yeast extract 1% malt extract 0.2% $CaCO_3$ artificial sea water “Coral Ocean”, ATI
H-medium (pH 7.4)	0.2% soy flour 0.2% glucose 0.8% starch 0.2% yeast extract 0.1% $CaCl_2$ 0.1% $MgSO_4$ 50 mM HEPES (11.8 g/L) 8 mg/L Fe-EDTA dest. water
ISP 2 (pH 7.0)	1% malt extract 0.4% yeast extract 0.4% glucose 1.5% Bacto Agar dest. water
ISP 3 (pH 7.2)	2% oatmeal (Quaker white oats) 1.8% Bacto Agar dest. water 1 mL/L Trace salt solution ISP3

ISP 4 (pH 7.3)	1% soluble starch 0.2% $(NH_4)_2SO_4$ 0.1% K_2HPO_4 0.1% $MgSO_4$ x 7 H_2O 0.1% NaCl 0.2% $CaCO_3$ 2% Bacto Agar dest. water
ISP 5 (pH 7.2)	0.1% L-Asparagine 1% glycerol 0.1% K_2HPO_4 1 mL/L Trace salt solution ISP 5 2% Bacto Agar dest. water
ISP 6 (pH 7.2)	1.5% peptone 0.5% proteose Peptone 0.05% C_6H_8FeNO 0.1% sodium glycerophosphate 0.0126% sodium thiosulfate-5-hydrate 0.1% yeast extract 2% Bacto Agar dest. water
ISP 7 (pH 7.3)	1.5% glycerol 0.05% L-Tyrosine 0.1% L-Asparagine 0.05% K_2HPO_4 0.05% NaCl 0.001% $FeSO_4$ x 7 H_2O 1 mL/L trace salt solution ISP 7 2% Bacto Agar dest. water
Marine Broth medium	Becton, Dickinson and Company, France
Middelbrock Broth medium	Becton, Dickinson and Company, France
Müller-Hinton Bouillon (MHB)	Carl Roth GmbH + Co.KG, Germany

Myc-medium (pH 7.0)	1% phytone peptone 1% glucose 50 mM HEPES (11.8 g/L) dest. water
Myxovirescin-medium (pH 7.0)	1% soy peptone 0.005% $CaCl_2$ 0.025% $MgSO_4$ 1 mg/L $CoCl_2$ 100 mM HEPES (23.8 g/L) dest. water
Sea water agar 0.8%	sampled sea water (NHS) 0.8% Bacto Agar
Sea water agar 1.5%	sampled sea water (NHS) 1.5% Bacto Agar
Solvent A (Maxis)	1 mL formic acid 1 L water
Solvent B (Maxis)	1mL formic acid 1 L acetonitrile
Stan 21	
solution A	3.2 g K_2HPO_4 6.4 mL yeast extract solution (1%) (from yeast extract micro granulate) 57.6 g Bacto Agar Addition of dest H_2O to 2.4 L
solution B	3.2 g KNO_3 3.2 g $MgSO_4$ x 7 H_2O 0.32 g $MnSO_4$ 1.08 g FeCl x 6 H_2O 3.2 mL trace element solution after Drewes 3.2 g $CaCl_2$ x 2 H_2O Addition of dest. H_2O to 0.8L
Combination of solution A and B	after autoclaving combination of solutions A and B at about 40 °C

Suter medium with and without tyrosine (pH 7.2)	1.5% glycerol (0.1% tyrosine) 0.5% L-arginine 0.5% L-glutamic acid 0.03% L-methionine 0.03% L-isoleucine 0.05% K_2HPO_4 $MgSO_4$ x 7 H_2O 1 mL/L trace element solution (5341) 2% Bacto Agar dest. water
Trace element solution 5314	0.3% $CaCl_2$ x H_2O 0.1% iron(III)citrate 00.2% $MnSO_4$ 0.01% $ZnCl_2$ 0.0025% $CuSO_4$ x 5 H_2O 0.02% $Na_2B_4O_7$ 0.0004% $CoCl_2$ x 6 H_2O 0.001% Na_2MoO_4 dest. water
Trace element solution 5341	1% $CuSO_4$ x 5 H_2O 0.1% $CaCl_2$ x 2 H_2O 0.1% $FeSO_4$ x 7 H_2O 0.1% $ZnSO_4$ x 7 H_2O 0.4% $MnSO_4$ x 7 H_2O dest. water
Trace salt solution ISP3	0.1 g/100 mL $FeSO_4$ x 7 H_2O 0.1 g/100 mL $MnCl_2$ x 4 H_2O 0.1 g/100 mL $ZnSO_4$ x 7 H_2O dest. water
Trace salt solution ISP 5	1 g/100 mL $FeSO_4$ x 7 H_2O 1 g/100 mL $MnCl_2$ x 4 H_2O 1 g/100 mL $ZnSO_4$ x 7 H_2O dest. water

Trypticase soy broth (TSB) (Soybean-Casein Digest Broth)	Becton, Dickinson and Company, France
VY/2 - ASW (pH 7.0)	0.5% yeast (fresh) 0.1% $CaCl_2$ 50 mM HEPES (11.8 g/L) 1.8% Bacto Agar artificial sea water “Coral Ocean”, ATI
Wadden Sea medium	10% supernatant of cooked Wadden Sea sediment 0.01% yeast extract dest. water
Wadden Sea sediment medium	50% Wadden Sea sediment 50% sampled sea water (NHS) 1.5% Bacto Agar
Water agar	dest. water 1.5% Bacto Agar
5010-medium (pH 6.8)	3% sucrose 0.2% $NaNO_3$ 0.1% KH_2PO_4 0.05% $MgSO_4$ x 7 H_2O 0.05% KCl 0.001% $FeSO_4$ dest. water
5038-medium (pH 7.2)	0.0857% KH_2PO_4 0.0125% K_2HPO_4 0.05% MgSO4 x 7 H_2O 0.01% NaCl 0.01% $CaCl_2$ x 6 H_2O 0.2% glucose 0.2% L-methionine 1 mL trace element solution 5314 dest. water
5080-medium (pH 7.2)	2% soy flour 2% mannit dest. water

5254-(ASW) medium (pH 7.2)	1.5% glucose 1.5% soy flour 0.5% corn steep liquor 0.2% $CaCO_3$ 0.5% NaCl (artificial sea water “Coral Ocean”, ATI)
5294-(ASW) medium (pH 7.2)	1% soluble starch 0.2% yeast extract 1% glucose 1% glycerol 0.25% corn steep liquor 0.2% peptone 0.1% NaCl 0.3% $CaCO_3$ (artificial sea water “Coral Ocean”, ATI)
5304-medium (pH 7.0)	0.1% glucose 2.4% starch 0.5% tryptone 0.3% meat extract 0.4% $CaCO_3$ dest. water
5305-medium (pH 7.0)	3% potato starch 1.5% soy flour 0.5% corn steep liquor 0.2% yeast extract 1 mL trace element solution 5314 dest. water
5319-medium (pH 7.0)	0.3% glycerol 0.2% casein peptone 0.1% K_2HPO_4 0.1% NaCl 0.05% $MgSO_4$ x 7 H_2O 5 mL trace element solution 5314 dest. water

5321-medium (pH 7.5)	1% peptone 2% glucose 0.2% $CaCO_3$ 0.0001% $CoCl_2$ x 6 H_2O dest. water
5330-medium (pH 6.5)	1% glucose 1% dextrin 0.5% yeast extract 0.5% casein peptone 0.1% $CaCO_3$ 0.5% celit dest. water
5333-medium (pH 7.0)	0.4% yeast extract 1.5% starch 0.1% K_2HPO_4 00.5% $MgSO_4$ x 7 H_2O dest. water
5334-medium (pH 6.9)	2% glucose 1% soy flour 0.02% $CaCO_3$ 0.0001% $CoCl_2$ x 6 H_2O dest. water
5336-ASW (pH 7.3)	1% soluble starch 0.1% casein 0.05% K_2HPO_4 0.5% $MgSO_4$ x 7 H_2O 2% Bacto Agar artificial sea water “Coral Ocean”, ATI
5388-medium (pH 6.8)	1.5% glycerol 1% soy flour 0.5% NaCl 0.1% $CaCO_3$ 0.0001% $CoCl_2$ dest. water

2.1.2. Chemicals

Table 2: Chemicals used in this study.

Chemicals	Manufacturer
1 kb DNA ladder	BioLabs
Acetone	J.T. Baker
Acetonitrile	J.T. Baker
Acetic acid	Roth
Ammonium acetate	Roth
Ammonium iron(III) citrate ($(NH_4)_5Fe(C_6H_4O_7)_2$)	Roth
Algea	Bamboo Garden Japan Nori-Blätter
Artificial sea water "Coral Ocean"	ATI, Hamm
Agarose	Gibco BRL
Arabinose	Merck
Bacto Agar®	BD
Chitin	Merck
Calcium chloride ($CaCl_2$ x $2H_2O$)	Merck
Calcium carbonate ($CaCO_3$)	Appli Chem Panreac
Casein peptone	Marcor
Cobalt(II) chloride (CoCl2)	Merck
Bacto Casitone	BD
Celit 503	Roth
Cellulose	Serva, Heidelberg
Corn steep liquor	Schering
Copper(II) sulfate ($CuSO_4$ x 5 H_2O)	Merck
Dimethylsulfoxid (DMSO)	Roth
DULBCCO´s modified EAGLE´s medium	DMEM, Bio Whittaker, Walkersville, MD
Ethylenediaminetetraacetic acid (EDTA)	Riedel-de Haën
Ethanol	J.T. Baker
Ethyl acetate	J.T. Baker
Iron(III) ethylenediaminetetraacetic acid (Fe EDTA)	Fluka
Fetal bovine serum (FBS)	FBS, JRH Bioscience, Lenexa, KS
Iron(III) chloride ($FeCl_3$ x $6H_2O$)	Merck
Iron(II) sulfate ($FeSO_4$ x 7 H_2O)	Riedel-de Haën
Formic acid	Sigma
Fructose	Roth
Glucose	Roth
Glycerol	Roth
Hydrochloric acid (HCL)	Roth
HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Roth
Inositol (Ino)	Merck
Iron(III) citrate ($FeC_6H_6O_7$ x H_2O)	Merck
Jump Start Taq Ready Mix	Stigma- Aldrich
Potassium chloride (KCl)	Stigma- Aldrich

Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Potassium hydrogen phosphate (K_2HPO_4)	Merck
Potassium nitrate (KNO_3)	Riedel-de Haën
L-asparagine	Merck
L-isoleucine	Fluka
L-glutamic acid	Merck
L-methionine	Merck
L-Tyrosine	Sigma
Mannitol	Merck
Marine Broth medium	Becton, Dickinson and Company, France
Meat extract	Roth
Malt extract	Roth
Methanol	J.T.Baker
Middelbrock Broth medium	Becton, Dickinson and Company, France
Magnesium sulfate ($MgSO_4x7H_2O$)	Roth
Manganes (II) chloride ($MnCl_2$)	Merck
Manganese sulfate (MnSO4)	Merck
Müller-Hinton Bouillon (MHB)	Carl Roth GmbH + Co.KG, Germany
Disodium tetraborade ($Na_2B_4O_7$ x 10 H_2O)	Merck
Sodium chloride (NaCl)	Merck
Sodium bromide (NaBr)	Merck
Sodium hydroxide (NaOH)	Riedel-de Haën
Ammonium sulfate ($(NH_4)_2SO_4$)	Merck
Sodium molybdate (Na_2MoO_4 x 10 H_2O)	Merck
Sodium nitrate ($NaNO_3$)	Merck
Oatmeal (Quaker white oats)	Herrnmühle
Peptone Marcor S	Marcor
Phytone peptone	Marcor
Potato starch	Denkavit Emsland
Proteose Peptone	BD
Rhamnose	Merck
Raffinose	Sigma
Skim milk powder	Merck
Soy flour	Cargill
Soy peptone	Merck
Sucrose	Merck
Starch	Roth
Soluble starch	Roth
Sodium glycerophosphate	Serva
Sodium thiosulfate-5-hydrate	Merck
SYBR-Green	Quiagen
Trypsin solution	Gibco
Trifluoro acetic acid	Riedel-de Haën
Trypticase soy broth (TSB) (Soybean-Casein Digest Broth)	Becton, Dickinson and Company, France

Tryptone	BD
Tyrosine	Sigma
XAD Amberlight 16N adsorber resin	Rohm and Hass
Xylose (Xyl)	Merck
Yeast extract	Ohly
Yeast extract micro granulate (yeast extract solution)	Carl Roth GmbH & Co. KG
Zinc chloride ($ZnCl_2$)	Merck
Zinc sulfate ($ZnSO_4$ x $7H_2O$)	Merck

2.1.3. Bacterial strains

All strains used in this study are listed below. Tables 3, 4, 5, 6 and 9 include the working strains such as the isolated myxobacteria from the Wadden Sea sediment and the marine actinobacteria isolated from sponges, marine sediment and mangroves from Guam and India as well as from deep sea sediments. Additionately, the different test panels used in this study are listed in tables 7 and 8. Furthermore, the strains used as reference strains for the characterization of the working strains were combined in table 9. Table 10 include the prey test organisms for the isolation of marine myxobacteria.

Table 3: Working strains: isolated myxobacteria from Wadden Sea sediment from the coast of Neuharlingersiel, Germany.

Name	Genus	Source	Method
WS 1.1	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea agar + *E.coli* (20°C)
WS 15.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (20°C)
WS 21.3	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea + *E.coli* (20°C)
WS 28.1	*Myxococcus*	Wadden Sea sediment NHS	Stan21 agar + filter paper (20°C)
WS 28.3	*Myxococcus*	Wadden Sea sediment NHS	Stan21 agar + filter paper (20°C)
WS 2.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (30°C)
WS 2.3	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (30°C)
WS 3.3	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea + *E.coli* (30°C)
WS 3.4	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea + *E.coli* (30°C)
WS 4.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + T4 (30°C)
WS 5.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (30°C)
WS 9.1	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea agar + *V. fischeri* (30°C)

Table 4: Working strains: actinobacteria isolated from sponges and sediment samples in Guam with and without Diffusion Growth Chamber (DGC).

Name	Genus	Source	Method
Guam 928	*Micromonospora*	Sponge (*Rhabdastrella globostellata*)	non-DGC
Guam 1509	*Micromonospora*	Sediment sample (Guam Blue Hole, 110m depth)	non-DGC
Guam 1510	*Micromonospora*	Sediment sample (Guam Blue Hole, 110m depth)	non-DGC
Guam 1582	*Micromonospora*	Sponge (*Rhabdastrella globostellata*)	DGC
Guam 1583	*Micromonospora*	Sediment sample (Guam Blue Hole, 110m depth)	non-DGC
Guam 1566	*Rhodococcus*	Sponge (*Rhabdastrella globostellata*)	DGC
Guam 1257	*Microbacterium*	Sponge (*Rhabdastrella globostellata*)	DGC
Guam 1322	*Streptomyces*	Sponge (*Rhabdastrella globostellata*)	DGC
Guam 1285	*Streptomyces*	Sponge (*Rhabdastrella globostellata*)	DGC

Table 5: Working strains: actinobacteria isolated from a marine sponge and sediment from a depth of 1092 m of the North Atlantic Ocean.

Name	Genus	Source	Method
ASO4 wet	*Streptomyces*	Sponge (sampled with ROV)	Stamping (wet)
A-Sed $H10^{-3}$	*Streptomyces*	Sediment (1092m depth)	Dilution series
B-Sed $H10^{-3}$	*Streptomyces*	Sediment (1092m depth)	Dilution series
C-Sed $H10^{-3}$	*Streptomyces*	Sediment (1092m depth)	Dilution series

Table 6: Working strains: Actinobacteria isolated from marine sediment and rhizosphere sediment samples from different mangrove plants from the coast of Tamil Nadu, India.

Name	Genus	Source
ICN4	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Avicinnia officinalis*)
ICN16	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Rhizophora mucronata*)
ICN18	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Rhizophora mucronata*)
ICN19	*Streptomyces*	Marine sediment
ICN21	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Rhizophora mucronata*)
ICN26	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Rhizophora mucronata*)
ICN27	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Rhizophora mucronata*)
ICN28	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Acrostichum aureum*)
ICN32	*Streptomyces*	Rhizosphere sediment of mangrove plant (*Rhizophora mucronata*)

Table 7: Standard test panel for the detection of the bioactivity of the secondary metabolites produced by the working strains.

Test strain	Strain no. / Comment
Staphylococcus aureus	Newman*
Bacillus subtilis	(DSM 10)
Micrococcus luteus	(DSM 1790)
Mycobacterium smegmatis	(ATCC700084)
Escherichia coli	(DSM 1116)
Escherichia coli	TolC
Pseudomonas aeruginosa	PA14 (DSM 19882)
Chromobacterium violaceum	(DSM 30191)
Candida albicans	(DSM 1665)
Mucor hiemalis	(DSM 2656)
Pichia anomaler	(DSM 6766)

* *Staphylococcus aureus* strain Newman was obtained from PD Dr. Markus Bischoff, Saarland University Hospital, Homburg (Duthie 1952).

Table 8: Multi resistant test panel for the detection of the bioactivity of secondary metabolites produced by the working strains.

Test strain / strain no.	Comment
Staphylococcus aureus Newman*	standard panel
Staphylococcus aureus N315 (DSM 11822)	MRSA; from Prof. Herrmann (Uniklinik Homburg)
Enterococcus faecium (DSM 20477)	Type strain microaerophilic
Enterococcus faecium (DSM 17050)	VREF (*vanA)* microaerophilic
Escherichia coli (DSM 1116)	standard panel
Escherichia coli WT-3	quinolone resistance, (#644; gyrA[S83L,D87G])
Escherichia coli XL-1 blue	tetracycline resistance, Tn10 (Tet^R) on F plasmid
Escherichia coli ESBL (DSM 22664)	produces CTX-M15 beta-lactamase
Pseudomonas aeruginosa PA14 (DSM 19882)	standard panel, from Prof. Häußler (Twincore)
Candida albicans (DSM 1665)	standard panel

* *Staphylococcus aureus* strain Newman was obtained from PD Dr. Markus Bischoff, Saarland University Hospital, Homburg (Duthie 1952).

Table 9: Test panel for the detection of the bioactivity of isolated pure compounds produced by the working strains.

Test strain / strain no.	Comment
Staphylococcus aureus Newman*	standard panel
Staphylococcus aureus N315 (DSM 11822)	MRSA; from Prof. Herrmann (Uniklinik Homburg)
Enterococcus faecium (DSM 20477)	Type strain microaerophilic
Enterococcus faecium (DSM 17050)	VREF (*vanA)* microaerophilic
Bacillus subtilis (DSM 10)	standard panel
Micrococcus luteus (DSM 1790)	standard panel
Mycobacterium sp. (DSM 43270)	standard panel
Escherichia coli (DSM 1116)	standard panel
Escherichia coli WT-3	quinolone resistance, (#644; gyrA[S83L,D87G])
Escherichia coli XL-1 blue	tetracycline resistance, Tn10 (Tet^R) on F plasmid
Escherichia coli ESBL (DSM 22664)	produces CTX-M15 beta-lactamase
Pseudomonas aeruginosa PA14 (DSM 19882)	standard panel, from Prof. Häußler (Twincore)
Chromobacterium violaceum (DSM 30191)	standard panel
Candida albicans (DSM 1665)	standard panel
Mucor hiemalis (DSM 2656)	standard panel
Pichia anomala (DSM 6766)	standard panel
Schizosaccharomyces pombe (DSM 70572)	standard panel
Rhodotorula glutinis (DSM 10134)	standard panel

* *Staphylococcus aureus* strain Newman was obtained from PD Dr. Markus Bischoff, Saarland University Hospital, Homburg (Duthie 1952).

Table 10: Reference strains for the phylogenetic characterization of the working strains (inclusive working strains *S. davawensis*, *S. cinnabarinus* and JS360 (*S. cinnabaragriseus*)).

Strain name	Strain no.
Streptomyces davawensis	JCM 4913 = DSM 101723^T
Streptomyces cinnabarinus	DSM 40467^T
Streptomyces cinnabaragriseus	JS360 = DSM 101724^T
Streptomyces avermitilis	DSM 46492^T
Streptomyces flavovariabilis	DSM 41479^T
Streptomyces novaecaesareae	DSM 40358^T
Streptomyces alboniger	DSM 40043^T
Streptomyces cellostaticus	DSM 40189^T
Streptomyces bobili	DSM 40056^T
Streptomyces galilaeus	DSM 40481^T
Streptomyces griseochromogenes	DSM 40499^T
Streptomyces pseudovenezuelae	DSM 40212^T
Streptomyces phaeoluteigriseus	DSM 41896^T
Streptomyces atriruber	DSM 41860^T
Streptomyces resistomycificus	DSM 40133^T
Streptomyces yokosukanensis	DSM 40224^T
Streptomyces olivochromogenes	DSM 40451^T

Streptomyces corchorusii	DSM 40340^T
Streptomyces longwoodensis	DSM 41677^T
Streptomyces curacoi	DSM 40107^T
Streptomyces antibioticus	DSM 40234^T
Streptomyces canus	DSM 40017^T
Streptomyces ciscaucasicus	DSM 40275^T
Streptomyces griseorubiginosus	DSM 40469^T
Streptomyces phaeopurpureus	DSM 40125^T
Streptomyces griseruber	DSM 40281^T
Streptomyces karpasiensis	DSM 42068^T
Streptomyces glycovorans	DSM 42021^T
Streptomyces abyssalis	DSM 42024^T
Streptomyces wuyuanensis	DSM 42132^T
Streptomyces indianensis	DSM 43803^T
Micromonospora chalcea	DSM 43026^T

Table 11: Prey organisms for the isolation of marine myxobacteria. All strains were isolated from Dr. Thorsten Brinkhoff (University of Oldenburg) from the marine environment.

	Species
TK	*Rhizobiales* sp.
T9	*Vibrio* sp.
T8	*Alteromonas* sp.
T17	*Oceanospirillum* sp.
BIA	*Flavobacte*r sp.
TV	*Cytophagales* sp.
Marinum T2	*Actinobacteria* sp.
T4	*Pseudonocardia* sp.
DR	*Dino roseobacter*

2.1.4. Kits

The listed Kits were used for the DNA isolation from myxobacteria and actinobacteria and the purification of the PCR products after 16S rRNA- PCR.

- PowerSoil®DNA isolation kit (MO Bio Laboratories Inc., USA)
- Invisorb Spin Plant Mini Kit (250) (stratec molecular, Germany)
- MasterPure^{TM} Yeast DNA Purification Kit (epicentre, Wisconsin, USA)

2.1.5. Primer

Used primer for the detection of the 16S rRNA (F27/R518/R1525) and the "Marine Myxobacteria Cluster" (MMC) sequence (MMC655f and MMC841r), respectively.

Table 12: Primer used in this study. Primer sets and the corresponding sequences: F27/R1525 (R518) for the detection of the 16S rRNA sequence and MMC655f/MMC841r to detect the MMC (F/f: forward primer; R/r: reverse primer).

Primer	Sequence	Reference
F27	AGAGTTTGATCMTGGCTCG	Czarnetzki & Tebbe 2004
R518	CGTATTACCGCGGCTGCTGG	Gafan et al. 2005
R1525	AAGGAGGTGATCCAGCCGCA	Czarnetzki & Tebbe 2004
MMC655f	AGTAATGGAGAGGGTGGC	Brinkhoff et al. 2012
MMC841r	GGCACAGCAGAGGTCAAT	Brinkhoff et al. 2012

2.1.6. Equipment

Table 13: Used equipment and manufacturers.

Equipment	Manufacturer
Centrifuge	Eppendorf Centrifuge 5804 R
Centrifuge	Eppendorf Centrifuge 5427 R
Clean Bench	Thermo Scientific Type MS 2020 1.2
CO_2 incubator	Thermo Scientific Heracell 150i CO_2 Incubator
HPLC (RP-HPLC system)	Agilent 1260 Series; Aligent technology, USA
HPLC	Aligent 1100 series; Aligent technology, USA
Incubator	Hereus Instruments Function Line
Light microscope	Zeiss Axio Sc pie. A1 microscope
MS (HRESIMS)	MaXis ESI-TOF-MS spectrometer (Bruker) equipped with an Agilent 1260 series RP-HPLC system
Multichannel pipette	RAININ 8-Kanal-Pipette ED P3 Plus 100-1200 µL
N_2 dryer (plates)	MiniVap (porvair science)
Photometer	IMPLEN Nano Photometer UV / VIS Spektralphotometer
Pipettes	Eppendorf Research plus
Rotary evaporator	Heidolph Laborata 4003
Shaker	Pilot-Shake System Kühner RC-6-U
Shaker (plates)	Heidolph Titramax 1000
Thermocycler	Eppendorf Thermocycler Mastercycler gradient
UV detector	Herolab RH-5.1 darkroom hood + B-1393-3K7N camera

2.2. Methods myxobacteria

2.2.1. Sampling

To isolate marine myxobacteria from marine sediment, Wadden Sea sediment was sampled at the North Sea (Germany). For a high distribution of the samples, we sampled at the coast of Neuharlingersiel, the island Juist and the sandbank Janssand (Figure 8).

Figure 8: Sampling sites of marine sediment for isolation of marine myxobacteria: the island Juist, the sandbank Janssand and the coast of Neuharlingersiel.
(Map: Maximilian Dörrbecker (https://en.wikipedia.org/wiki/East_Frisian_Islands))

2.2.1.1. Neuharlingersiel

At the coast of Neuharlingersiel the first samples were collected at low tide next to the coast. Sediment samples from the upper two centimetres as well as snail shells were collected. Furthermore, for the isolation of anaerobe and facultative anaerobe marine myxobacteria, sediment cores from the Wadden Sea down to the depth of about 15 cm from different locations (mud, close to the beach and close to the water) were taken (Figure 9).

Figure 9: Wadden Sea sediment cores from the coast of Neuharlingersiel (northern Germany) from different locations: mud, close to the beach and close to the water

2.2.1.2. Juist

Because of the high similarity of the isolated "marine" *Myxococcus* sp. strains to terrestrial ones (Brinkhoff et al. 2012) samples were also taken from islands to minimise the probability to isolate terrestrial strains which were distributed by humans walking around the beach. Five different samples from the island Juist were collected by Thorsten Brinkhoff from the University of Oldenburg: dry sand from the beach, wet sand from the beach saturated with sea water, wet algae (green): *Ulva* sp., wet algae (brown): *Fucus* sp. and a dried algae (brown) *Fucus* sp. (Figure 10).

Figure 10: Samples for the isolation of myxobacteria from the island Juist, Germany: 1: dry sand from the beach; 2: wet sand from the beach saturated with sea water; 3: algae (green): *Ulva* sp., algae (brown): *Fucus* sp.; 4: dried algae (brown) *Fucus* sp..

2.2.1.3. Janssand

To further minimize the ability for wash-in or drag-in of terrestrial bacteria by humans into the marine sediment samples, the sandbank "Janssand" near Spiekerook (Figure 11) was sampled. This sandbank is well known in the field of marine research. The upper two centimetres of the sediment on different locations as well as rock worm excretions were taken at low tide for the following isolation of aerobic bacteria.

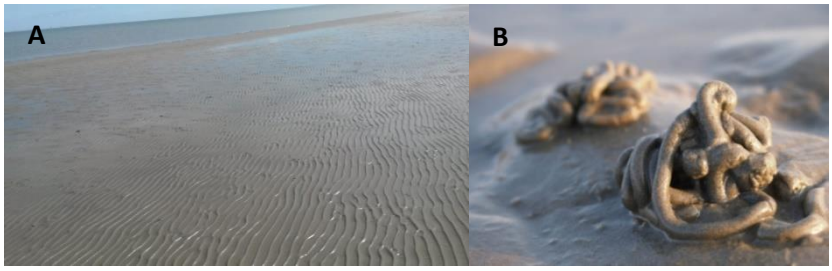


Figure 11: Sampling site Janssand. A: Sandbank Janssand and B: example of rock worm excretions sampled for the isolation of new myxobacteria.

2.2.2. Isolation of marine myxobacteria

To isolate myxobacteria from marine sediments four different media were used. Like Reichenbach described in the Prokaryotes (Shimkets et al. 2006) water agar treated with dead *E. coli* dots as well as Stan 21 agar, picked with filter paper, were utilized to isolate the predatory as well as the cellulose degrading myxobacteria, respectively. To enrich especially the marine myxobacteria, the isolation media were modified. Collected seawater from the cost of the North Sea was used for seawater agar plates. Subsequently, Wadden Sea media was established which was made up out of the supernatant from cooked marine sediment. Another approach was to prepare Wadden Sea sediment plates which were made up out of 50% sampled sediment from the Wadden Sea from Neuharlingersiel and 50% collected sea water. All approaches were treated with *E. coli* dots to isolate the predatory myxobacteria. Furthermore, Wadden Sea sediment Agar plates were picked with filter paper to isolate cellulose degraders. Afterwards, the different samples, were placed on every prey dot or filter paper and incubated at room temperature, at 30°C as wells as at 30 °C with 5% CO_2, respectively. The sediment cores were split up and separated into 1 cm sections. For the isolation centimetres 1, 4, 8 and 12 were used.

Furthermore, coated agar plates were prepared. Therefore, some inoculated sea water agar plates were coated with 0.8% of sea water agar to mimic the semi anoxic conditions close underneath the sediment surface. To create a more natural environment for the isolation of the predatory marine myxobacteria different prey organisms from the natural habitat were used. The Wadden sea and water agar media were treated with *E. coli* dots and crosses as well as some marine prey organisms (TK (*Rhizobiales* sp.), T9 (*Vibrio* sp.), T8 (*Alteromonas* sp.), T17 (*Oceanospirillum* sp.), BIA (*Flavobacter* sp.), TV (*Cytophagales* sp.), marinum T2 (*Actinobacteria* sp.), T4 (*Pseudonocardiacea* sp.) and *Dino Roseobacter* strain (DR)). Afterwards, the plates were incubated at room temperature and at 30 °C.

Moreover, a mesocosm was constructed inside an aquarium which was filled with sediment from the Wadden Sea as well as sampled sea water. To enrich the myxobacteria, some coated agar slides (2% agar, 5% peptone) were placed close to the sediment. After an incubation time of five days, the agar was transferred to different enrichment plates described before (Brinkhoff et al. 2012).

2.2.3. DNA isolation

2.2.3.1. DNA isolation from sediment samples and algae

The DNA was isolated from the sediment samples from the Wadden Sea from Neuharlingersiel, Juist and Janssand as well as from the different algae samples. An old sediment sample from Bensersiel was used as positive control. For the isolation the PowerSoil®DNA isolation kit (MO Bio Laboratories Inc., USA) was used. About 0.25 g of the sediment or a small piece of algae was applied and the procedure was done like manufacturers description.

2.2.3.2. DNA isolation from cells for 16S rRNA analysis and detection of the MMC sequence

The DNA was isolated via Invisorb Spin Plant Mini Kit (250) (stratec molecular, Germany). For preparation either, 1.5 mL of al well grown cell suspension was centrifuged or some cells were taken from an agar plate. The cell pellet was mixed with 100 µL of lysation buffer and incubated at 95 °C for 5 min. Afterwards, 300 µL lysation buffer and 20 µL protein kinase K were added, mixed up and incubated for 30 min at 65 °C. Further DNA isolation steps were done like manufactures description.

2.2.4. Molecular approaches

2.2.4.1. Specific PCR for the detection of the MMC

The isolated DNA from the sediment samples and algae was diluted 1:10 for the MMC PCR. The specific primer MMC655f and MMC841r (Brinkhoff et al. 2012) were used. The PCR mixture had a final volume of 25 mL and consisted of the fallowing components: water, “Jump Start Ready Mix” (JSRM), the primer pair MMC655f/MMC8415r as well as the DNA template. The JSRM is a mixture including Jump Start Taq DNA polymerase, 99% pure desoxynucleotides and buffer in an optimized reaction concentration. The PCR mixture had a final volume of 25 µL and consisted of the fallowing components: 12.5 µL JSRM, 10 µL water, 1 µL forward and reverse primer dilution as well as 0.5 µL of the template DNA. The conditions for the PCR were based on a publication by Brinkhoff et al. (2012). To find the best conditions it was modified to the following protocol for a touch down PCR. The PCR within the thermocycler started with an activation temperature of 95 °C for 5 min. The first cycle of the touch down PCR started with a denaturation step of 94 °C for 1 min and a primer annealing at 70 °C for 1 min, followed by two minutes of elongation at 72 °C. During the next nine cycles the annealing temperature was decreased for 1 °C per cycle to 60 °C. Finally, for the replication of the

template, 28 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 2 min were performed.

Afterwards, the samples were mixed with loading buffer charged with DNA-dye SYBR-Green and were loaded on a 0.8% agarose gel. After 60 min at 70 V the bands were visible under UV light.

2.2.4.2. 16S rRNA PCR for phylogenetic analysis

To analyze the 16S rRNA sequence, two primers were used matching most of the known eubacterial orders on the positions 27 (forward) and 518 (reverse). For the conduction of the 16S rRNA PCR a special mastermix was created, containing water, primers and "Jump Start Ready Mix" (JSRM). The PCR mixture had a final volume of 50 µL and consisted of the fallowing components: 25 µL JSRM, 22 µL water, 1 µL forward and reverse primer dilution as well as 1 µL of the template DNA. The PCR within the Thermocycler started with an activation temperature of 95 °C for 5 min. Followed by 34 cycles of denaturation (94 °C, 30 sec.), a primer annealing at 52 °C for 30 sec. and 2 min of elongation at 72 °C. The final elongation was done at 72°C for 10 minutes. Afterwards, the samples were mixed with loading buffer charged with the DNA-dye SYBR-Green and were loaded on a 0.8% agarose gel. After 90 min at 70 V the bands were detectible under UV light. Finally, all samples were sequenced und compared to bacterial type strain sequences using the NCBI "BLAST" tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

2.2.4.3. Calculation of phylogenetic trees using ARB

The 16S rRNA sequences of the isolated myxobacterial or actinobacterial strains, respectively, as well as the 16S rRNA sequences of the closest related type strains detected via NCBI "Blast" (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) were imported into the ARB database (Ludwig et al. 2004; version 14.02.2005 database; http://www.arbhome.de). After the alignment, a distance matrix tree was constructed using the Neighbour-Joining method (Saitou & Nei 1987) and Jukes Cantor correction (Jukes & Cantor 1969). The topology of the phylogenetic tree was built by bootstrap analysis of 1000 operons without using a filter.

The ARB tree for the myxobacterial part was constructed by Dr. Kathrin Mohr (working group Microbial Drugs, HZI Braunschweig).

2.2.5. Sodium chloride tolerance

Because of the marine habitat of the isolated myxobacteria from the Wadden Sea, their resistance towards sodium chloride was tested. The tolerance was analyzed by the growth of the strains on basal medium with the addition of 0, 2.5, 5, 7.5 and 10 % of sodium chloride. After five to ten days of incubation the highest salt concentration still allowing growth was detected.

2.2.6. Screening for secondary metabolites

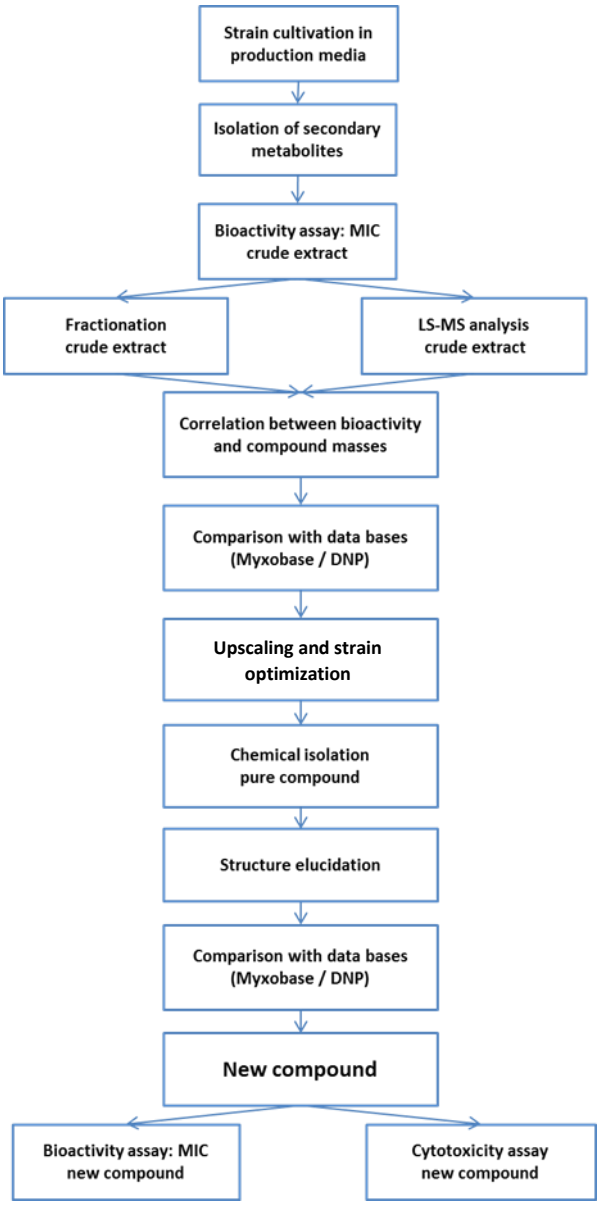


Figure 12: Flow chart of the screening procedure used in this study.

Figure 12 gives an overview about the different steps of the screening process. All methods are described in detail in the following chapters. To screen for secondary metabolites, the strains were cultivated in production media and afterwards the secondary metabolites were isolated. With the

help of a bioactivity assay to detect the MIC (Minimal Inhibition Concentration) via serial dilution assay on a standard and a multi resistant test panel, antifungal and antibiotic activities of the crude extracts (mixture of compounds produced by the organism) were detected. Crude extracts which show a high or interesting biological activity, e. g. activities against *Candida albicans*, *Pseudomonas aeruginosa* or multi resistant bacteria, were chosen for further analysis. To identify the bioactive compound in the mixture of different compounds, the crude extract was fractionated via semi-preparative HPLC into a 96-well plate which was dried and inoculated with the positive tested pathogens. Simultaneously the crude extract was analyzed via LC-MS to detect the masses of all compounds within the mixture. Correlating the LC-MS data with the results of the HPLC fractionation, the wells which show growth inhibitions can be assigned to the corresponding peak in the LC-MS chromatogram revealing the mass of the biologically active compound. Due to a search in the in-house database Myxobase (Krug & Müller 2014) and the dictionary of natural products (DNP) most of the hits were correlated to known compounds. If a compound could not be identified, its production was enhanced by optimization of the culture conditions. Using the optimized culture conditions the culture is scaled up and the biologically active compound was isolated. Structure elucidation was done via HRESIMS spectrometry and NMR spectroscopy. A structure search was conducted, using the Myxobase, the DNP as well as SciFinder and other open access chemical databases, ensuring the novelty of the isolated compounds. The antibiotic activity and the cytotoxicity of the pure compounds were tested to determine their respective activity spectra.

2.2.6.1. Extraction of secondary metabolites

The 15 isolated myxobacteria (20 °C: WS 1.1, WS 15.1, WS 21.3, WS 28.1 WS 28,3; 30 °C: WS 2.2, WS 2.3, WS 3.3, WS 4.1, WS 5.1, WS 9.1) were cultured in 100 mL E-medium, CY/H-medium and Myxovirescin-medium, treated with 2% of the absorber resin Amberlight XAD-16. After 14 days of incubation at 20 °C and 30 °C, respectively, the XAD was reaped. The XAD and the cells were filtered through a metal mesh and flushed with dest. H_2O. Subsequently, the resin was dried and removed to a flask, 70 µL of acetone were added and the resulting mixture was incubated for one hour. Afterwards, the acetone extract was filtrated into a 250 mL round bottom flask and the solvent was evaporated in a rotary evaporator at 40 °C. The resulting residue was resolved in 1 mL methanol.

2.2.6.2. Bioactivity test via serial dilution assay with standard panel

Table 14: Test strains and the corresponding medium and incubation temperatures of the standard test panel used for the bioactivity tests of crude extracts.

Test strain	Medium	Temp.
Staphylococcus aureus Newman*	MHB	30°C
Bacillus subtilis (DSM 10)	MHB	30°C
Micrococcus luteus (DSM 1790)	MHB	30°C
Mycobacterium smegmatis (ATCC700084)	Middlebrock	37°C
Escherichia coli (DSM 1116)	MHB	37°C
Escherichia coli TolC	MHB	37°C
Pseudomonas aeruginosa PA14 (DSM 19882)	MHB	37°C
Chromobacterium violaceum (DSM 30191)	MHB	30°C
Candida albicans (DSM 1665)	Myc	30°C
Mucor hiemalis (DSM 2656)	Myc	30°C
Pichia anomala (DSM 6766)	Myc	30°C

* *Staphylococcus aureus* strain Newman was obtained from PD Dr. Markus Bischoff, Saarland University Hospital, Homburg (Duthie 1952).

To test the bioactivity of the crude extracts, a panel of Gram-positive and -negative bacteria as well as fungi and yeasts were used (Table 14). The assays were conducted according to standard procedures using 96-well plates. Initially, an OD_{600} of 0.01 (for yeast and fungi 0.05) of the test strains was adjusted in 20 mL of the suitable media listed in table 14. Subsequently, 150µl of the test strain culture was pipetted in every well, merely the first row was inoculated with 130µl. Afterwards, 20µl of each extract was adjoined to the wells of the first row. The last well (A12) was used as negative control by adding 20µl of methanol. The extract was diluted in 1:2 steps down to row H and the last 150µl were discarded. Finally, the plates were incubated for 1-2 days at 30°C and 37°C, respectively (Table 14).

2.2.6.3. Compound analysis: HPLC fractionation and mass spectrometry (MS)

Fractionations were carried out using an analytical HPLC (High performance liquid chromatography) (Aligent 1100 series; Agilent technology, USA) system equipped with a fraction collector. Using HPLC, different chemical compounds could be detected in a liquid mixture. Diverse agents can be distinguish by means of the time they need to elute from the column. This time is called “retention time” and is individual for every substance. The fractionation of the extracts helps to identify the substance which inhibits a certain test organism. While the HPLC measurement, the detached substances from the column were collected in a 96-well plate. Every 30 seconds the injector switched to another well and thereby the retention time of the different substances can be correlated with the

specific wells and afterwards with the inhibition of the test organisms. The fractionations were carried out with an Agilent 1100 HPLC system equipped with a diode-array UV detector (DAD) and a fraction collector. HPLC conditions: XBridge C_{18} column 100×2.1 mm (Waters), 3.5 µm, eluent A2: H_2O-acetonitrile (95/5), 5 mmol NH_4Ac, 0.04 mL/L CH_3COOH; eluent B2: H_2O-acetonitrile (5/95), 5 mmol NH_4Ac, 0.04 mL/L CH_3COOH; gradient system: 10% B increasing to 100% B in 30 min; flow rate 0.3 mL/min; 40 °C; UV-detection at 210-450 nm.

The samples in the 96-well plates were dried for 45-60 min at 40°C with heated nitrogen in a MiniVap (porvair science). Afterwards, 150 µL of the specific test organism were added to each well and the inoculated plates were incubated at 30/37 °C for 24 h. For the inoculation mixture 20 mL of a suitable medium were inoculated with the test organism to a final OD_{600} of 0.01 (for yeast and fungi 0.05). Specific media and incubation temperatures can be taken out of tables 14 and 15.

The MS analysis in this study was carried out via HPLC-MS (High Pressure Liquid Chromatography – Mass Spectrometry) or in detail via HPLC-HRESIMS (High Pressure Liquid Chromatography – high resolution electron spray ionization mass spectrometry). Thus, the HPLC which separate the different chemical compounds is coupled to a mass spectrometer to identify and quantify the substances via mass to charge ratio m/z. Within this combination, every substance was correlated to the corresponding peak in the HPLC run. Therefore, the HRESIMS data were recorded on a MaXis ESI-TOF-MS spectrometer (Bruker) equipped with an Agilent 1260 series RP-HPLC system. HPLC system: Acquity UPLC BEH C_{18} column 50x2.1 mm (Waters); solvent A: 0.1% formic acid in H_2O, B: 0.1% formic acid in acetonitrile; gradient system: 5% B for 0.5 min, in 19.5 min to 100% B and holding 5 min at 100% B; flow rate 0.6 mL/min; 40 °C; UV-detection at 200-600 nm. Alternatively with buffer gradient system: XBridge C_{18} column 100×2.1 mm (Waters), 3.5 µm, solvent A: H_2O-acetonitrile (95/5), 5 mmol NH_4Ac, 0.04 mL/L CH_3COOH; solvent B: H_2O-acetonitrile (5/95), 5 mmol NH_4Ac, 0.04 mL/L CH_3COOH; gradient system: 10% B increasing to 100% B in 30 min; flow rate 0.3 mL/min; 40 °C; UV detection at 200-600 nm. Molecular formulae were calculated using the SmartFormula algorithm including the isotopic pattern (Bruker).

2.3. Methods actinobacteria

2.3.1. Isolation of actinobacteria from marine deep sea sediment from the North Atlantic Ocean

2.3.1.1. Sampling

The samples were taken by the Team of Prof. Dr. Peter Schupp (University of Oldenburg) in December 2014 from the North Atlantic Ocean during an expedition of the new marine research vessel “Sonne”. They sampled two sponges and one soft-coral via ROV as well as marine sediment from 1092 m depth.

Figure 13: Sampling of the marine samples taken by the research vessel “Sonne”. A: Sampling site; B: sampling of the sponge and C: sampling of the sediment sample via ROV.

2.3.1.2. Isolation

For the isolation of marine actinobacteria from deep sea sediment different isolation media were prepared as agar plates: VY/2–ASW, water agar treated with dead *E.coli*, GYM-ASW and 5336-ASW. To inoculate the plates the team of Prof. Dr. Schupp used various isolation techniques: dilution series of sediment samples with autoclaved sampled sea water; heat treatment of homogenated sampled organisms up to 60 °C before plating them in different dilution series; as well as wet and dry stamping of sampled organisms. Therefore, the organism was stamped with a previously raised area several times on the agar plate to cause a dilution. Afterwards the plates were incubated for 8-10 weeks at about 18 °C. Colonies, which seemed to be actinobacteria were picked with a sterile inoculating loop and streaked on a fresh GYM-ASW plate.

2.3.2. Cultivation

2.3.2.1. Cultivation of actinobacterial strains

For the cultivation of the nine strains from Guam, two different media were used. Guam 928, 1509, 1582 and 1583 were cultured in GYM medium. The remaining strains, Guam 1285, 1510, 1566, 1257 and 1322 showed better growth in Marine Broth medium. The nine strains from India, ICN4, ICN16, ICN18, ICN19, ICN21, ICN26, ICN27, ICN28, ICN32 as well as strains ASO4 wet, A-, B- and C-Sed $H10^{-3}$ from the deep sea sediment from the North Atlantic Ocean were cultured in GYM medium. Additionally, strains JS360, *S. cinnabarinus*, *S. davawensis* and all further *Streptomyces* strains used in this study were cultured in GYM medium.

All strains were cultured in 250 mL flasks filled with a volume of 100 mL at 30 °C and 160 rpm for 5 to 8 days.

2.3.2.2. Cultivation for the extraction of secondary metabolites

For the extraction of secondary metabolites, the isolated actinobacteria were cultured in the production media 5254 and 5294 with and without sampled sea water or artificial sea water. All strains were cultured in 250 mL flasks filled with a volume of 100 mL at 30 °C and 160 rpm.

2.3.3. Characterization

To characterize the *Streptomyces* strains, the morphology, physiology, phylogeny and chemotaxonomy as well as the production of secondary metabolites has been analyzed. In this study we concentrated on the parts listed in figure 14. The chemotaxonomic analyses were done by the working group of Karin Martin of the HKI in Jena and for the DNA-DNA hybridization the isolated DNA was sent to the working group of Prof. Dr. Peter Kämpfer, University of Gießen (Germany).

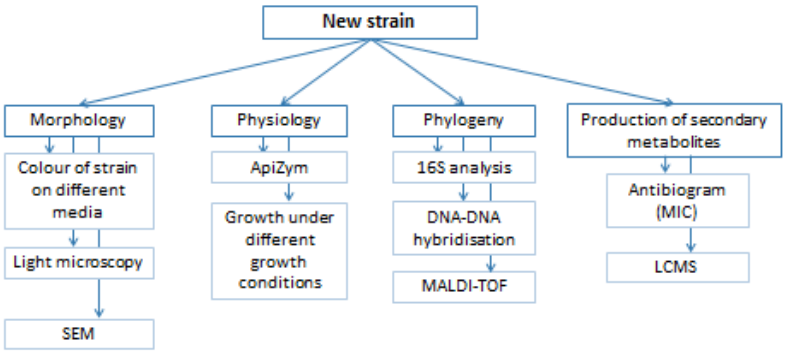


Figure 14: Flow chart of the aspects mentioned for the isolation of a new strain: morphology, physiology, phylogeny and the production of secondary metabolites.

2.3.3.1. Morphology

2.3.3.1.1. Growth on agar plates

For the analysis of the morphology and colony description, the strains were plated on different media described by Shirling and Gottlieb (1966). Therefore, about 500 µL of a well grown liquid culture was plated on different agar plates: The *Streptomyces* medium GYM, yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7). Furthermore, the last two ISP media as well as Suter medium (Suter 1978) with and without tyrosine were used for the detection of melanin production which is visible as a dark brown to black pigment in the agar (Kutzner 1981). After the incubation time of 10-14 days at 30 °C (or any other temperature which is required by the strain) the morphological differences like growth, colony colour (detected via RAL-code colour cards), areal mycelium and soluble pigments were observed and correlated to the closest related type strains.

2.3.3.1.2. Light and electron microscopy (SEM)

For the light microscopic images one drop of a 5-day old liquid culture was taken with an inoculation loop and the slide was regarded under a 400-fold amplification with a Zeiss Axio Sc pie. A1 microscope.

To detect the structure of mycelium and spores, electron microscopy was used. Therefore, the cultures were grown on agar plates of complex media like GYM or the ISP 3 oatmeal medium. According to the description of Wink (2003), a well-covered piece was cut out and fixed in glutaraldehyde. After critical-point-dehydration and gold-palladium-sputtering, a Zeiss Merlin field emission scanning electron microscope (SEM) was used to observe diverse spores and spore chains. The SEM harbour an Everhart-Thornley SE-detector and Inlens-SEM detector in a 25 : 75 % ratio applying the SEMSmart software version 5.05.

The electron microscopic analyses were done by Prof. Dr. Manfred Rhode, HZI Braunschweig.

2.3.3.2. Physiology

2.3.3.2.1. Utilization of carbohydrates

The ability to utilize different carbon sources is a marker for the differentiation of bacteria. To get an overview of the utilization of carbon sources of the different actinobacteria, their growth was detected on minimal medium with the addition of different carbohydrates used as source. The experiment was performed in 12 well plates filled with basal medium including 1% of the fallowing carbon sources: glucose (Glu), arabinose (Ara), sucrose (Suc), xylose (Xyl), inositol (Ino), mannitol (Man), fructose (Fra), rhamnose (Rha), raffinose (Raf) and cellulose (Cel). Additionately, water was taken as negative and glucose as positive control (Shirling & Gottlieb (1966)). After the inoculation of about 100 µL of a well grown liquid culture of the test strain, the plates were incubated 10-14 days at 30 °C (or any other temperature which is required by the strain).

2.3.3.2.2. Sodium chloride tolerance

The resistance towards sodium chloride is a helpful tool for the differentiation between all species of actinobacteria not only between those of the marine or halophilic representatives. Therefore, basal medium agar was prepared with addition of 0, 2.5, 5, 7.5 and 10% of sodium chloride and filled in 6 well plates. The different media were inoculated with about 100 µL of a well grown liquid culture of the test strain and incubated 10-14 days at 30 °C (or any other temperature which is required by the strain). The highest salt concentration still allowing growth was recorded (Kutzner, 1981).

2.3.3.2.3. Enzymatic activity via api® stripes

ApiZym® stripes were developed by BioMérieu for the identification of microorganisms by determining a specific fingerprint of enzymatic activities (Humble *et al*. 1977). In 1978 Kilian demonstrated the successfully identification of Actinomycetales and related bacteria after using this method. The method was performed as described by the manufacturer. To detect an enzymatic reaction a homogenized suspension of a well grown liquid culture is transferred into the test stripes which contain different substrates. In case of an enzymatic reaction, a visible colour can be detected. Using the ApiZym® stripes the reaction of the following enzymes and substrates can be detected: phosphatase alkaline: 2-naphthyl phosphate , esterase (C 4): 2-naphthyl butyrate, esterase lipase (C 8): 2-naphthyl caprylate, lipase (C 14): 2-naphthyl myristate, leucine arylamidase: L-leucyl-2-naphthylamide, valine arylamidase: L-valyl-2-naphthylamide, cystine arylamidase: L-cystyl-2-naphthylamide, trypsin: *N*-benzoyl-DL-arginine-2-naphthylamide, chymotrypsin: *N*-glutaryl-phenylalanine-2-naphthylamide, phosphatase acid: 2-naphthyl phosphate, naphthol-AS-BI-phosphohydrolase: Naphthol-AS-BI-phosphate, galactosidase: 6-Br-2-naphthyl-D-galactopyranoside, galactosidase: 2-naphthyl-D-galactopyranoside, glucuronidase: Naphthol-AS-BI-D-glucuronide, glucosidase: 2-naphthyl-D-glucopyranoside, glucosidase: 6-Br-2-naphthyl-D-glucopyranoside, *N*-acetyl-glucoseamidase: 1-naphthyl-*N*-acetyl-D-glucoseaminide, mannosidase: 6-Br-2-naphthyl-D-mannopyranoside and fucosidase: 2-naphthyl-L-fucopyranoside.

2.3.3.3. Phylogeny

2.3.3.3.1. DNA isolation and 16S rRNA PCR for the taxonomic description

The genomic DNA for the phylogenetic analysis was isolated with the MasterPure™ Yeast DNA Purification Kit (epicentre, Wisconsin, USA). The isolation was done as described by the manufacturer.

The 16S rRNA analyses were done as described in chapter 2.2.4.2 for the myxobacteria. For the actinobacterial strains the primer set F27 and R1525 was used.

2.3.3.3.2. Calculation of phylogenetic trees using the Genome-to-Genome Distance Calculator (GGDC) of the DSMZ

Pairwise sequence similarities were calculated using the method recommended by Meier-Kolthoff et al. (2013b) for the 16S rRNA gene available via the GGDC web server (Meier-Kolthoff et al. 2013a) available at http://ggdc.dsmz.de/.Phylogenies were inferred by the GGDC web server (Meier-Kolthoff et al. 2013a) available at http://ggdc.dsmz.de/ using the DSMZ phylogenomics pipeline (Meier-Kolthoff et al. 2014) adapted to single genes. A multiple sequence alignment was created with MUSCLE (Edgar 2004). Maximum likelihood (ML) and maximum parsimony (MP) trees were inferred from it with RAxML (Stamatakis 2014) and TNT (Goloboff et al. 2008), respectively. For ML, rapid bootstrapping in conjunction with the autoMRE bootstopping criterion (Pattengale et al. 2010) and subsequent search for the best tree was used; for MP, 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates. The sequences were checked for a compositional bias using the X^2 test as implemented in PAUP* (Swofford 2002).

All data was unrooted and used for an overview about the relationship between the isolated strains and their closest relatives.

2.3.3.3.3. MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) analysis

The sample preparation was done according to the ethanol/formic acid extraction protocol described by Schumann & Maier (2014): about 10 mg of biomass from a liquid culture (cultivated in GYM medium for 6-8 days at 30 °C or the optional temperature of the individual strain) was suspended in 300 µL H_2O and homogenised carefully. 900 µL ethanol was added to the suspension. Further preparation steps as well as the MALDI-TOF measurements and following analyses were done at the DSMZ in Braunschweig at the working group of Dr. Peter Schumann.

MALDI-TOF mass spectra were recorded using a Microflex L20 mass spectrometer (Bruker Daltonics) with a N2 laser unit. The spectra were measured in linear positive mode and the acceleration voltage was 20 kV. The spectra were collected from 250 shots across a spot. To analyze the data, a mass range of m/z 2000-20,000 Da was observed. For internal calibration the Bacterial Test Standard #255343 (Bruker Daltonics) was used. Using the Flexanalysis software (version 3.3, Bruker Daltonics), the MALDI-TOF MS spectra were smoothed, baseline corrected and re-calibrated. A score-orientated dendrogram was calculated by using the BioTyper software (version 3.1, Bruker Daltonics).

2.3.3.3.4. RiboPrinter® analysis

RiboPrinter® analysis was used to distinguish strains of the same species. Based on ribosomal sequences on the DNA this method generates a significant fingerprint for every analyzed strain after fragmentation of the DNA using specific enzymes. The experimental part was carried out as described by Schumann & Pukall (2013).

The sample preparation and the following workflow inside the fully automated Characterization Unit of the RiboPrinter® system is divided into 5 parts: sample preparation (outside of the Charaterization Unit), DNA preparation, separation and transfer, membrane processing and pattern detection. For the sample preparation, a small amount of cells from a well grown GYM medium agar was used. The RiboPrinter® analysis was conducted by the working group of Dr. Peter Schumann, DSMZ (Germany).

2.3.4. Production of secondary metabolites

2.3.4.1. Screening of secondary metabolites

The screening procedure (2.2.6.1), the bioactivity test (serial dilution assay for the detection of the MIC) with the standard panel (2.2.6.2) as well as the compound analysis via HPLC fractionation and mass spectrometry (MS) (2.2.6.1.3) were done as described in the myxobacterial chapters.

2.3.4.1.1. Extraction of secondary metabolites

20 mL of a 5-day old culture was mixed with 20 mL of ethyl acetate in a 50 mL reaction tube. After a 2 min shaking step the tubes were mixed for 10 min in a rotary shaker. Afterwards the samples were centrifuged at 9000 rpm for 10 min and the upper phase was transferred into a 50 mL round bottom flask. At about 40 °C the ethyl acetate is evaporated in a rotary evaporator. Finally, the extract was solved in 1 mL of ethyl acetate: acetone: methanol (1:1:1).

2.3.4.1.2. Bioactivity tests of crude extracts via serial dilution assay with multi resistant test panel

To confine the strains for further analysis, a new serial dilution assay test panel with mainly multi resistant bacteria was created. For this panel bacteria with common antibiotic resistances and their corresponding type strains (Table 15) were used. The procedure of the serial dilution assay was done as described above for the standard test panel. Aside from *Candida albicans* (OD_{600}: 0.05, 30 °C) all test strains were inoculated at an OD_{600} of 0.01 and were cultured at 37 °C.

Table 15: Test strains and the corresponding medium and incubation temperatures multi resistant test panel used for the bioactivity tests of crude extracts.

Test strain	Comment	Medium	Temp.
Staphylococcus aureus Newman*	standard panel	MHB	37 °C
Staphylococcus aureus N315 (DSM 11822)	MRSA; from Prof. Herrmann (Uniklinik Homburg)	MHB	37 °C
Enterococcus faecium (DSM 20477)	Type strain microaerophilic	TSB	37 °C
Enterococcus faecium (DSM 17050)	VREF (*vanA)* microaerophilic	TSB	37 °C
Escherichia coli (DSM 1116)	standard panel	MHB	37 °C
Escherichia coli WT-3	quinolone resistance, (#644; gyrA[S83L,D87G])	MHB	37 °C
Escherichia coli XL-1 blue	tetracycline resistance, Tn10 (TetR) on F plasmid	MHB	37 °C
Escherichia coli ESBL (DSM 22664)	produces CTX-M15 beta-lactamase	MHB	37 °C
Pseudomonas aeruginosa PA14 (DSM 19882)	standard panel, from Prof. Häußler (Twincore)	MHB	37 °C
Candida albicans (DSM 1665)	standard panel	MYC	30 °C

* *Staphylococcus aureus* strain Newman was obtained from PD Dr. Markus Bischoff, Saarland University Hospital, Homburg (Duthie 1952).

2.3.4.2. Optimization of culture conditions: Media variation, kinetics and "upscaling"

For the extraction of secondary metabolites, the strains were cultured in the production media 5254 and 5294 with and without artificial sea water ("Coral Ocean", ATI). To enhance the production of interesting compounds the culture conditions were optimized.

Strain Guam 1582 was cultured in 5294 medium with dest. water, artificial sea water ("Coral Ocean", ATI) and sampled sea water form Neuharlingersiel. The peptone was varied between Bacto Peptone (BP) and Marcor Soy Peptone (PSM). For comparison, a fresh activated strain as well as a strain which was inoculated several times was used.

Additionally to the media 5254 and 5294, strain Guam 928 was cultivated in the following media: 5010, 5038, 5080, 5288, 5304, 5305, 5319, 5321, 5330, 5333 and 5334.
A second media variation for the strain Guam 928 was done using the screening medium 5294 with the addition of some compounds of the marine habitat such as shells, algae, chitin, NaBr as well as cobalt chloride. Eight variations of medium 5294 without artificial sea water were also used, each omitting one component of the original medium.
Every experiment was done in 250 mL flasks filled with 100 mL of the respective medium. The cultures were inoculated 1:10 with a 5 day old well grown preculture and incubated at 30 °C and 160 rpm for 7 days.

For the strains Guam 1582 and 928 a kinetic experiment was conducted to find the optimal growth period for the best production of the compound. Six 250 mL flasks filled with 100 mL of 5294-ASW medium, each, were inoculated 1:10 with a 5 day old well grown preculture and were incubated at

30 °C and 160 rpm. From day 3 to 8 every day one flask was harvested and crude extract was produced. For strain Guam 928 only two flasks were inoculated and harvested at days 5 and 7.
For the isolation of interesting compounds 50 250 mL flasks, filled with 100 mL of the medium showing the best metabolite production, were inoculated 1:10 with a 5 day old preculture of a well grown strain. Afterward the cultures were incubated at 30 °C and 160 rpm for 7 days.

2.3.4.3. Isolation, purification and structure elucidation of pure compounds

These experiments were conducted by Sabrina Karwehl, PhD student in the working group "Microbial drugs" of the HZI, Braunschweig (Germany).

2.3.4.4. Bioactivity tests of pure compounds: serial dilution assay and cytotoxicity test

The biological activity was tested using a serial dilution assay to detect the minimal inhibition concentration (MIC) of the pure compounds. The procedure was done as described in Kuhnert et al. 2015. The assay was done in a 96-well plate. The isolated bioactive compounds were solved in DMSO (Dimethylsulfoxid) in a 1 mg/mL concentrated stock solution. This solution was mixed with the test strain (OD_{600}: 0.01 (bacteria) / 0.05 (yeast) / OD_{548}: 0.1 (*Schizosaccharomyces pombe*; *Rhodotorula glutinis*)) to a final concentration of 66.6 µg/mL in the first lane and was diluted in 1:2 steps with the inoculated test strain medium. In addition to the standard panel consisting of yeasts, fungi, Gram-positive and -negative bacteria, a test panel consisting of multi resistant strains, e. g. MRSA und VREF (Table 16) was used. The strains were cultivated in the corresponding medium and after inoculation the plates were incubated at 30/37 °C and 160 rpm for one day.

Table 16: Description, media and incubation temperature of the strains of the test panel for the analysis of pure compounds.

Test strain	Comment	Medium	Temp.
Staphylococcus aureus Newman*	standard panel	MHB	37 °C
Staphylococcus aureus N315 (DSM 11822)	MRSA; from Prof. Herrmann (Uniklinik Homburg)	MHB	37 °C
Enterococcus faecium (DSM 20477)	Type strain microaerophilic	TSB	37 °C
Enterococcus faecium (DSM 17050)	VREF (*vanA)* microaerophilic	TSB	37 °C
Bacillus subtilis (DSM 10)	standard panel	MHB	37 °C
Micrococcus luteus (DSM 1790)	standard panel	MHB	30 °C
Mycobacterium sp. (DSM 43270)	standard panel	MHB	30 °C
Escherichia coli (DSM 1116)	standard panel	MHB	37 °C
Escherichia coli WT-3	quinolone resistance, (#644; gyrA[S83L,D87G])	MHB	37 °C
Escherichia coli XL-1 blue	tetracycline resistance, Tn10 (Tet^R) on F plasmid	MHB	37 °C
Escherichia coli ESBL (DSM 22664)	produces CTX-M15 beta-lactamase	MHB	37 °C
Pseudomonas aeruginosa PA14 (DSM 19882)	standard panel, from Prof. Häußler (Twincore)	MHB	37 °C
Chromobacterium violaceum (DSM 30191)	standard panel	MHB	30 °C
Candida albicans (DSM 1665)	standard panel	MYC	30 °C
Mucor hiemalis (DSM 2656)	standard panel	MYC	30 °C
Pichia anomala (DSM 6766)	standard panel	MYC	30 °C
Schizosaccharomyces pombe (DSM 70572)	standard panel	MYC	30 °C
Rhodotorula glutinis (DSM 10134)	standard panel	MYC	30 °C

* *Staphylococcus aureus* strain Newman was obtained from PD Dr. Markus Bischoff, Saarland University Hospital, Homburg (Duthie 1952).

To determine the cytotoxicity of the substances the murine fibroblast cell line L929 was used as target in a serial dilution assay in 96 well plates to detect the minimal inhibition concentration (MIC). The cells were cultured in tissue culture flasks in culture medium consisting of DULBCCO's modified EAGLE's medium (DMEM, Bio Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS). For the cytotoxicity assay the cells were removed from the flask using 0.25% of a trypsin solution (Gibco). After removing the trypsin, the cells were incubated for 5 min at 37 °C and 5% CO_2. The detached cells were resuspended in 10 mL of the culture medium DMEM + 10% FBS and the concentration of 50,000 cells/mL were adjusted. 87 µL of culture medium was inserted in the first wells of the 96 well plate. After the addition of 3 µL of the compounds, the substances were diluted in 1:6 steps with the medium stock solution. Subsequently, 120 µL of the previously prepared L292 cell line stock solution (50,000 cells/mL) was appended to every well. The cells were incubated for 5 days at 37 °C at 5% CO_2. For the analysis the cells were examined under a light microscope.

3. Results

3.1. Part 1: Isolation of marine myxobacteria from the sediment of the Wadden Sea: search for the MMC sequence and screening for new bioactive compounds

All samples were tested for the presence of the MMC sequence. Simultaneously, myxobacteria were isolated and afterwards also screened for the MMC sequence. Furthermore, the samples were phylogenetically analyzed, the sodium chloride tolerance of the isolates was tested and they were screened for the production of new bioactive secondary metabolites.

3.1.1. Isolation of new myxobacteria from marine samples

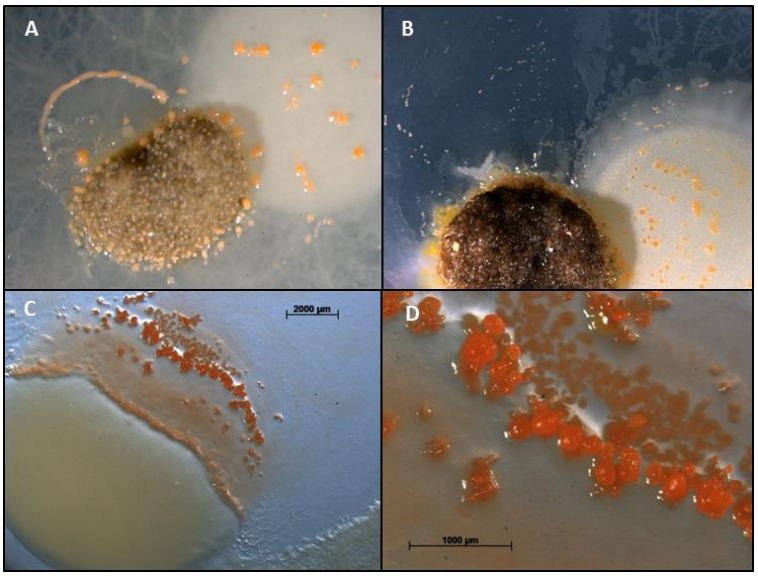


Figure 15: Isolates of the marine sediment samples from the Wadden Sea. A and B: sediment samples on *E. coli* dot and visible fruiting bodies and swarming of myxobacteria. C and D: Isolated myxobacterium *Myxococcus sp.* WS 3.4: C shows the lysis of an *E.coli* dot by the *Myxococcus* strain WS 3.4 and D the fruiting bodies in detail.

After 10 days of incubation the first myxobacterial fruiting bodies and swarming were visible. The first colonies were detected at the wet sediment samples on the Wadden Sea agar plates and water agar plates with *E. coli* as prey organisms which were incubated at 30 °C (Figure 15 A, B). At the end of this study 12 *Myxococcus* strains were isolated from Wadden Sea and water agar plates treated with *E. coli* dots as well as the Wadden Sea isolates T4 (*Pseudonocardiaceae sp.*) and *Vibrio fischeri* and from Stan 21 plates. 5 of these *Myxococcus* strains were isolated and afterwards incubated at 20 °C (WS 1.1, 15.1, 21.3, 28.1, 28.3) and the remaining 7 strains (WS 2.1, 2.3, 3.3, 3.4, 4.1, 5.1, 9.1) at 30 °C (Table 17). All strains were visible in different shades of orange (example Figure 15 C, D). Some strains form some kind of inhibition zone around their swarming area to protect themselves from the fungi on the enrichment plates.

Table 17: Name, genus, source and isolation method from the new isolated strains from Wadden Sea sediment: WS 1.1, 15.1, 21.3, 28.1, 28.3, 2.1, 2.3, 3.3, 3.4, 4.1, 5.1 and 9.1.

Name	Genus	Source	Method
WS 1.1	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea agar + *E.coli* (20 °C)
WS 15.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (20 °C)
WS 21.3	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea + E.*coli (*20 °C)
WS 28.1	*Myxococcus*	Wadden Sea sediment NHS	Stan21 agar + filter paper (20 °C)
WS 28.3	*Myxococcus*	Wadden Sea sediment NHS	Stan21 agar + filter paper (20 °C)
WS 2.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (30 °C)
WS 2.3	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (30 °C)
WS 3.3	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea + E.*coli (*30 °C)
WS 3.4	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea + E.*coli (*30 °C)
WS 4.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + T4 (30 °C)
WS 5.1	*Myxococcus*	Wadden Sea sediment NHS	Water agar + *E.coli* (30 °C)
WS 9.1	*Myxococcus*	Wadden Sea sediment NHS	Wadden Sea agar + *V.fischeri* (30 °C)

The strain Juist 2.2 was isolated from the wet sand from the beach saturated with sea water from the island Juist. With its yellow colour, swarming growth and rod shape this bacterium looked like a myxobacterium (Figure 16). However, the sequencing results showed that the isolated bacterium was a *Cellulophaga lytica* which belongs to the family *Flavobacteriaceae* within the phylum *Bacteroidetes.* This bacterium was previously isolated from an alga (*Fucus*) from a marine habitat (Johansen *et al*. 1999). This fact supports the assumption that the yellow isolates which were isolated from algae samples from Juist belong to the same family.

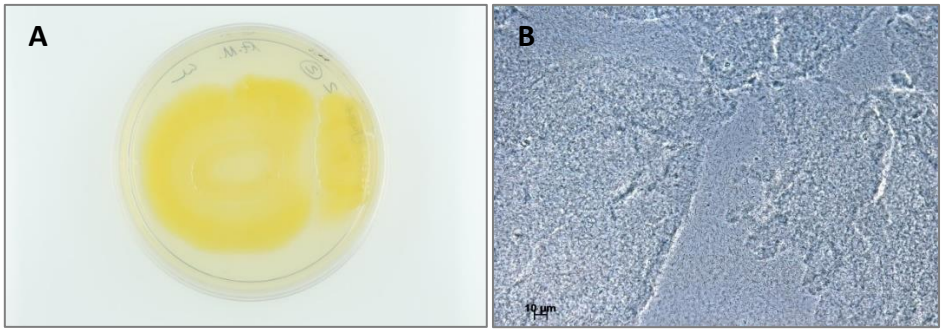


Figure 16: Isolate Juist 2.2: *Cellulophaga lytica*. A: Strain growing on a Marine Broth medium plate; B: Microscopic image of a well grown culture in Marine Broth medium

During the work of the bachelor thesis of Stephanie Akgün, no differences between the used marine prey organisms and the standard *E. coli* were detected. During the enrichment processes swarming bacteria were picked but to date there is no pure culture available. The analysis of the whole genomic DNA from the sediment samples showed the MMC sequence detection in the coastal sediment samples of Neuharlingersiel as well as in the samples taken from the sandbank Janssand.

From all of the other samples no myxobacterial pure culture was isolated.

3.1.2. Detection of the MMC sequence in the different samples and the isolated myxobacteria from the Wadden Sea

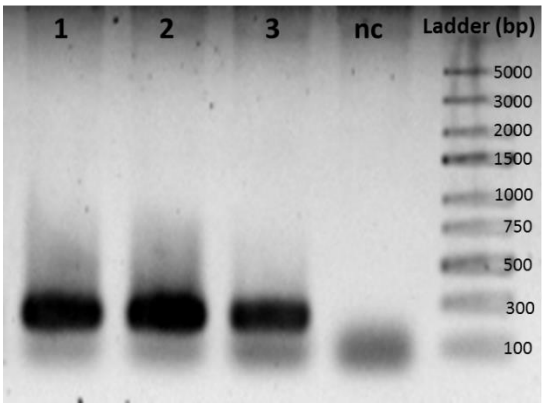


Figure 17: Image of the agarose gel of the specific MMC PCR 1. 1: positive control sediment Bensersiel; 2, 3: sediment sample Neuharlingersiel

On the PCR gel a clear band was observed in the positive control as well as in the two sediment samples from Neuharlingersiel (Figure 17). Furthermore, the MMC sequence was detected in all depths of the Wadden Sea sediment cores from Neuharlingersiel (appendix), in the different sediment and rock worm excrution samples from Janssand (appendix), in the wet sediment samples from Juist (Figure 18, sample 2) and Neuharlingersiel (Figure 18, sample 6-9) as well as in the snail shells (Figure 18, sample 5). However it was not found in one sediment core sample from a depth of 12 cm (appendix) as well as the dry sand from Neuharlingersiel, in all of the algae samples (Figure 18, sample 3b, 3g, 4, 10, 11) and the dry sand from Neuharlingersiel (Figure 18, sample 1).

A positive signal confirms the existence of living or dead organisms in the sediment samples which harbour the MMC sequence.

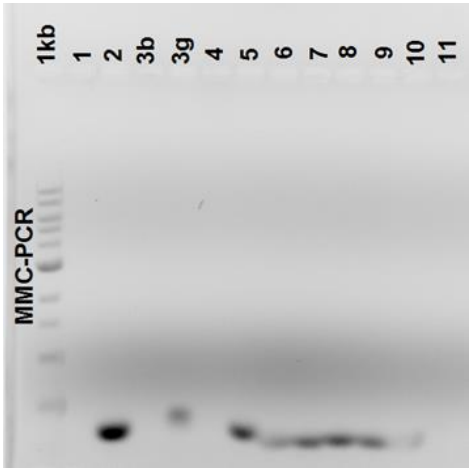


Figure 18: Image of the agarose gel of the specific MMC PCR 2. 1: dry sand from the beach (Juist), 2:wet sand from the beach saturated with sea water (Juist), 3b: algae brown: *Fucus* (Juist), 3g: algae green: *Ulva* (Juist), 4: dried algae (*Fucus*) (Juist), 5: snail shells (NHS), 6: light Wadden Sea sediment (NHS), 7: dark /green Wadden Sea sediment (NHS), 8: dark /brown-black Wadden Sea sediment (NHS), 9: Wadden Sea sediment near the coast (NHS), 10: light green algae wet, 11: dark green algae wet.

All 12 isolated *Myxococcus* strains, listed in table 17 were tested due to the existence of the marine myxobacteria sequence in their genome. Unfortunately, the MMC sequence was not detected in any of the new isolated strains, resulting none of them belong to the “Marine Myxobacteria Cluster”.

3.1.3. Phylogeny of isolated myxobacteria

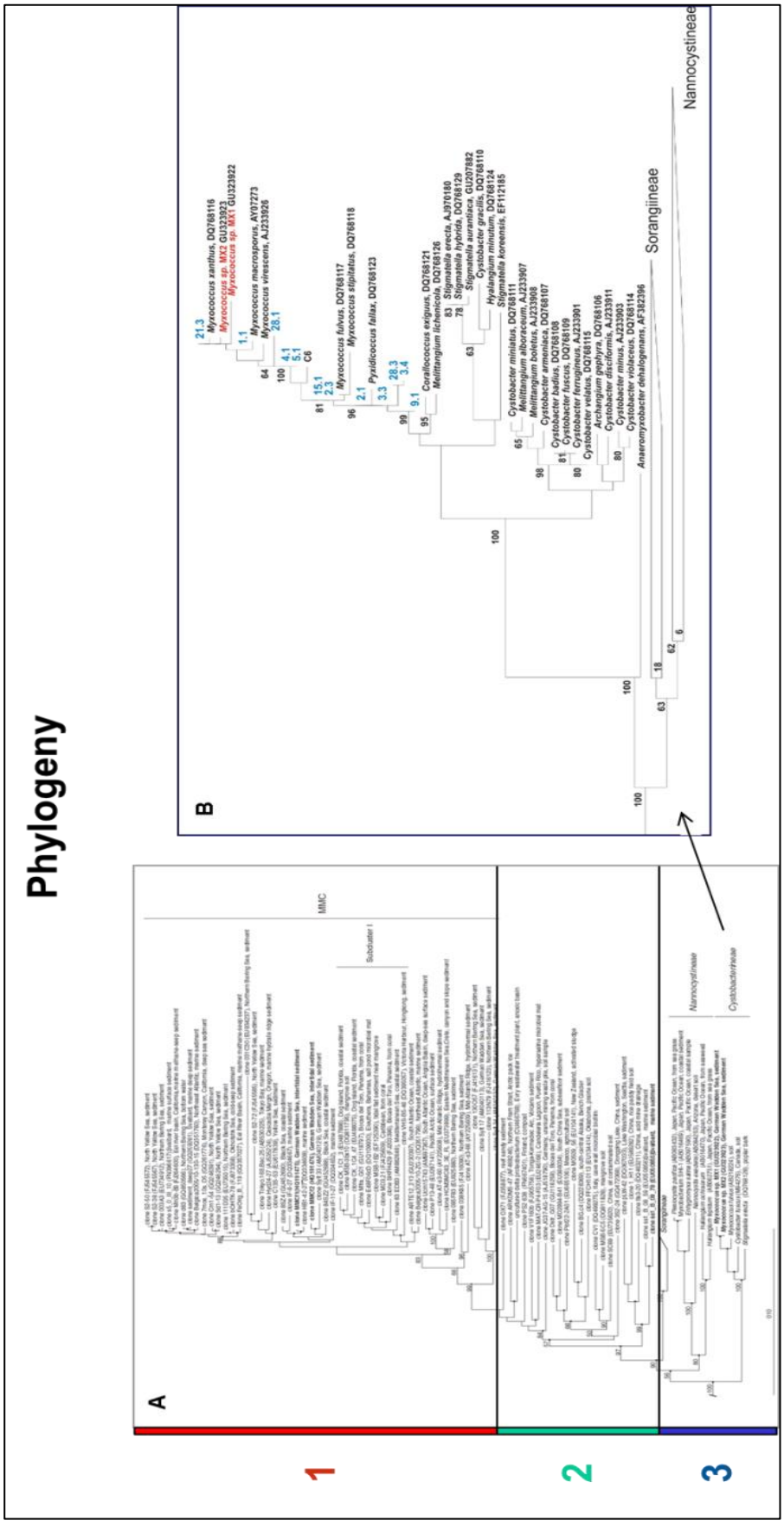


Figure 19: Phylogeny of isolated myxobacteria. A: Neighbour-joining tree showing the phylogenetic relationships of bacteria of the MMC within the Myxococcales based on 16S rRNA gene sequence similarity. 1: "Marine Myxobacteria Cluster" (MMC); 2: Cluster of uncultured myxobacteria from extreme habitats; 3: Cultured marine myxobacteria including the isolated *Myxococcus sp.* isolated from Wadden Sea sediment (Brinkhoff et al. 2012). B: Neighbour-joining tree showing the phylogenetic relationships of the 12 Myxococcus sp. strains (WS 1.1, 2.1, 2.3, 3.3, 3.4, 4.1, 5.1, 9.1, 15.1, 21.1, 23.1, 28.1, 28.3) isolated from Wadden Sea sediment in this study and the myxobacterial type strains based on 16S rRNA gene similarity.

The 16S rRNA sequences of the 12 *Myxococcus* isolates were clustered against the marine myxobacterial clones (1: red cluster) and uncultured myxobacteria from extreme habitats (2: green) as well as the cultured myxobacterial type strains (3: blue) (modified after Brinkhoff et al. 2012). The isolates clustered very close to the cultured *Myxococcus* type strains but also to the two *Myxococcus* isolates MX1 and MX2 which were isolated before from Brinkhoff et al. (2012) from the University of Oldenburg (Figure 19).

3.1.4. Sodium chloride tolerance of the isolated myxobacteria

The growth of the different strains was detected by the phenotypic colour of the fruiting bodies and the swarm. All strains grew well at 0% NaCl. Swarming behavior and formation of orange fruiting bodies were detected. At higher salt concentrations, the fruiting bodies were colourless und the cells did not seem to be very fit (Table 18, Figure 20). The strain WS 5.1 showed a good growth and orange fruiting bodies at salt concentrations up to 10% (Table 18).

Table 18: Sodium chloride tolerance of the isolated *Myxococcus sp.* strains from the Wadden Sea: o fb: orange fruiting body; c fb: colourless fruiting bodies.

Strain	0% NaCl	2.5% NaCl	5% NaCl	7.5% NaCl	10% NaCl
WS 1.1	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 2.1	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 2.3	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 3.3	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 3.4	o fb, swarm	o fb, swarm	cl fb	cl fb	cl fb
WS 4.1	o fb, swarm	o fb, swarm	cl fb	cl fb	cl fb
WS 5.1	o fb, swarm	o fb, swarm	o fb	o fb	o fb
WS 9.1	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 15.1	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 21.1	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 23.1	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 28.1	o fb, swarm	cl fb	cl fb	cl fb	cl fb
WS 28.3	o fb, swarm	cl fb	cl fb	cl fb	cl fb

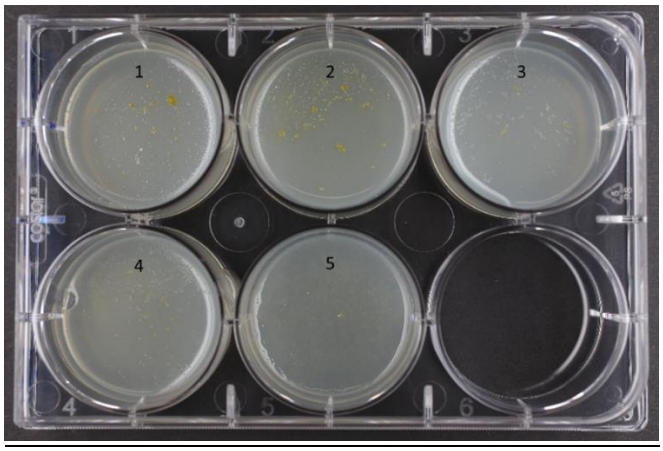


Figure 20: Sodium chloride tolerance test plate of the isolated *Myxococcus sp*. strain WS 4.1. 1: 0% NaCl, 2: 2.5% NaCl, 3: 5% NaCl, 4: 7.5% NaCl, 5: 10% NaCl.

3.1.5. Secondary metabolites produced by the isolated myxobacteria

The 12 isolated myxobacteria from Wadden Sea sediment were cultivated in the three production media Myxovirescin, CY/H and E medium. These media are used especially for members of the genus *Myxococcus*. After cultivation and isolation of the secondary metabolites, the bioactivity was tested via serial dilution assay to detect the MIC. Interesting compounds were detected by analytical scale fractionation experiments and identified using LC-MS analysis.

3.1.5.1. Bioactivity tests: serial dilution assay with standard test panel

After cultivation and harvesting of the secondary metabolites the bioactivity of the crude extract was tested on the standard test panel.

Table 19: MIC of the crude extracts from the *Myxococcus* strains, isolated from the Wadden Sea sediment, in different media: [1] Myxovirescin; [2] CY/H medium; [3] E medium.

	Fungi			**Gram +**				**Gram -**			
	P. anomala (DSM 6766)	*C. albicans* (DSM 1665)	*M. hiemalis* (DSM 2656)	*M. luteus* (DSM 20030)	*B. subtilis* (DSM 10)	*S. aureus* Newman*	*M. phlei* (DSM 43070)	(*C. violaceum* DSM 30191)	*P. aeruginosa* (DSM 50071)	*E.coli* (DSM 1116)	*E.coli* TolC
WS 1.1[1]	-	-	A	-	E	C	B	-	-	-	A
WS 1.1[2]	-	-	A	-	G	E	D	-	-	-	B
WS 1.1[3]	-	-	-	-	D	D	A	-	-	-	A
WS 2.1[1]	C	A	A	A	C	C	A	-	-	-	A

WS 2.1[2]	-	-	A	D	F	E	E	-	-	-	C
WS 2.1[3]	-	-	A	A	C	C	A	-	-	-	A
WS 2.3[1]	C	A	B	A	C	D	A	-	-	-	B
WS 2.3[2]	-	-	A	B	E	D	D	-	-	-	B
WS 2.3[3]	-	-	A	A	C	C	A	-	-	-	A
WS 3.3[1]	C	A	B	A	C	C	-	-	-	-	A
WS 3.3[2]	-	-	A	B	F	D	D	-	-	-	B
WS 3.3[3]	-	-	A	A	C	C	A	-	-	-	A
WS 3.4[1]	-	-	-	-	-	B	D	-	-	-	-
WS 3.4[2]	-	-	A	A	E	D	-	-	-	-	B
WS 3.4[3]	-	-	A	-	A	B	-	-	-	-	-
WS 4.1[1]	C	A	-	A	B	C	F	A	-	-	G
WS 4.1[2]	-	-	-	A	F	E	D	-	-	-	G
WS 4.1[3]	-	-	A	A	C	B	-	-	-	-	E
WS 5.1[1]	C	B	F	H	H	H	A	H	-	E	H
WS 5.1[2]	-	-	-	E	G	G	-	D	-	-	G
WS 5.1[3]	-	-	-	E	F	G	D	D	-	-	F
WS 9.1[1]	C	A	A	A	B	C	A	A	-	-	A
WS 9.1[2]	-	-	-	A	E	D	-	-	-	-	B
WS 9.1[3]	-	-	-	-	A	B	-	-	-	-	-
WS 15.1[1]	-	-	A	-	E	C	B	-	-	-	-
WS 15.1[2]	-	-	A	-	F	E	C	-	-	-	B
WS 15.1[3]	-	-	-	-	D	C	A	-	-	-	-
WS 21.3[1]	-	-	A	-	H	C	B	-	-	-	A
WS 21.3[2]	-	-	A	-	H	E	C	-	-	-	A
WS 21.3[3]	-	-	-	-	E	C	B	-	-	-	-
WS 28.1[1]	-	-	A	F	G	G	D	D	-	A	E
WS 28.1[2]	-	-	A	H	H	H	C	G	-	B	G
WS 28.1[3]	-	-	-	H	H	G	-	D	-	A	E
WS 28.3[1]	-	-	A	-	G	E	D	-	-	-	B
WS 28.3[2]	-	-	-	-	E	C	B	-	-	-	A
WS 28.3[2]	-	-	-	-	D	C	-	-	-	-	-

Most of the extracts showed moderate biological activities (Table 19). Concentrating on strains showing growth inhibitions higher than a dilution step of 1:8 (row C) for further fractionation experiments, mostly compounds that were already known could be found.

3.1.5.2. Known compounds produced by the isolated myxobacteria

Most of the produced compounds of the isolated marine myxobacteria showed a high bioactivity against Gram-positive bacteria (Table 19). This growth inhibition is caused by the production of fatty acids like octadecadienoic acids (e. g. linoleic acid) or hexadecenoic acids (e. g. palmitoleic acid). The production of fatty acids is well known for myxobacteria especially for *Myxococcus* sp. species. The correlation between the bioactivity of the fatty acids to the growth inhibition on the test plate is demonstrated with the example of strain WS 1.1 in CY/H medium (Figure 21).

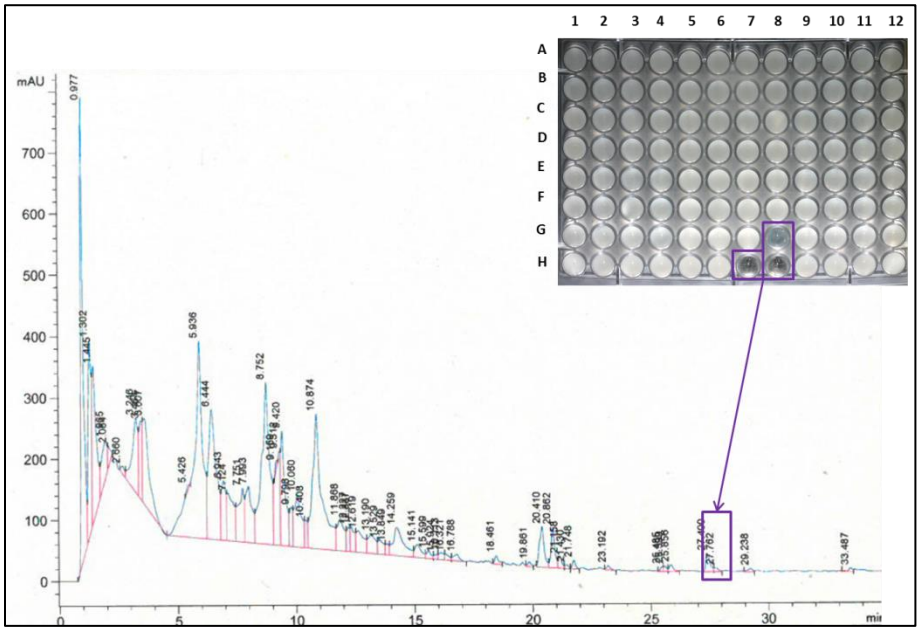


Figure 21: Correlation between the bioactivity of the extract of WS 1.1 in CY/H medium against the test organism *E.coli* and the corresponding peak in the HPLC chromatogram.

In addition to the bioactivity against Gram-positive bacteria, fatty acids as well as other media compositions and cell components could also cause a weak growth inhibition of the Gram–negative bacterium *E. coli* Tol C.

3.1.5.3. HPLC fractionation and MS analysis of the crude extract of strain *Myxococcus* sp. WS 5.1

The MIC from the crude extract of strain WS 5.1 in Myxovirescin medium, revealed a strong activity against *E.coli*. To identify the responsible compound for the inhibition, the extract was fractionated and afterwards inoculated with *E.coli*. The growth inhibition of the *E.coli* strain was correlated to a peak in the HPLC chromatogram with the retention time of 10.9 min (Figure 22). After LC-MS analysis, this peak (*m/z* 567.2444 $[M+H]^+$) was identified as the known compound saframycin (saframycin Mx1 BCH) (Trowitzsch-Kienast et al. 1988) (Figure 23). Saframycin Mx1 BCH is described as to show a strong activity against *E.coli*. Additionally a saframycin derivative with a saframycin-chromophore and a specific chlorine isotopic pattern was detected (Figure 24). Unfortunately, this saframycin derivative could not be reproduced.

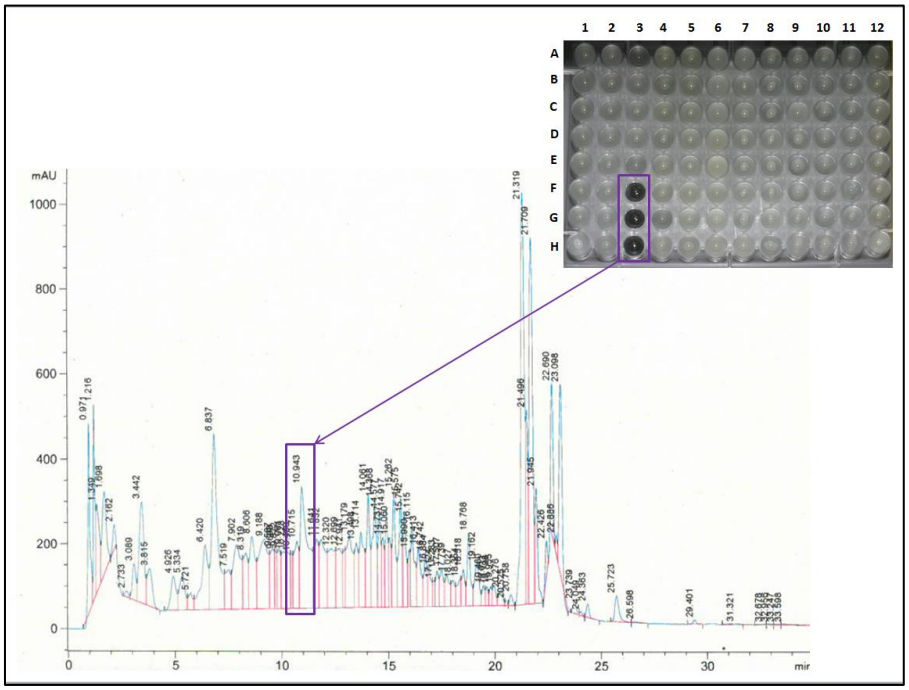
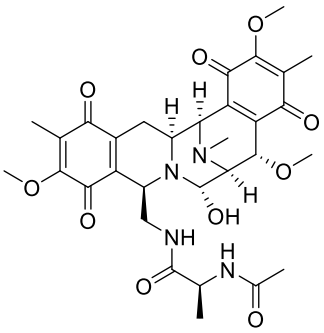


Figure 22: Correlation between the bioactivity of the extract of WS 5.1 in Myxovirescin medium against the test organism *E.coli* and the corresponding peak in the HPLC chromatogram.



Saframycin Mx1 BCH

Figure 23: Chemical structure of saframycin Mx1 BCH.

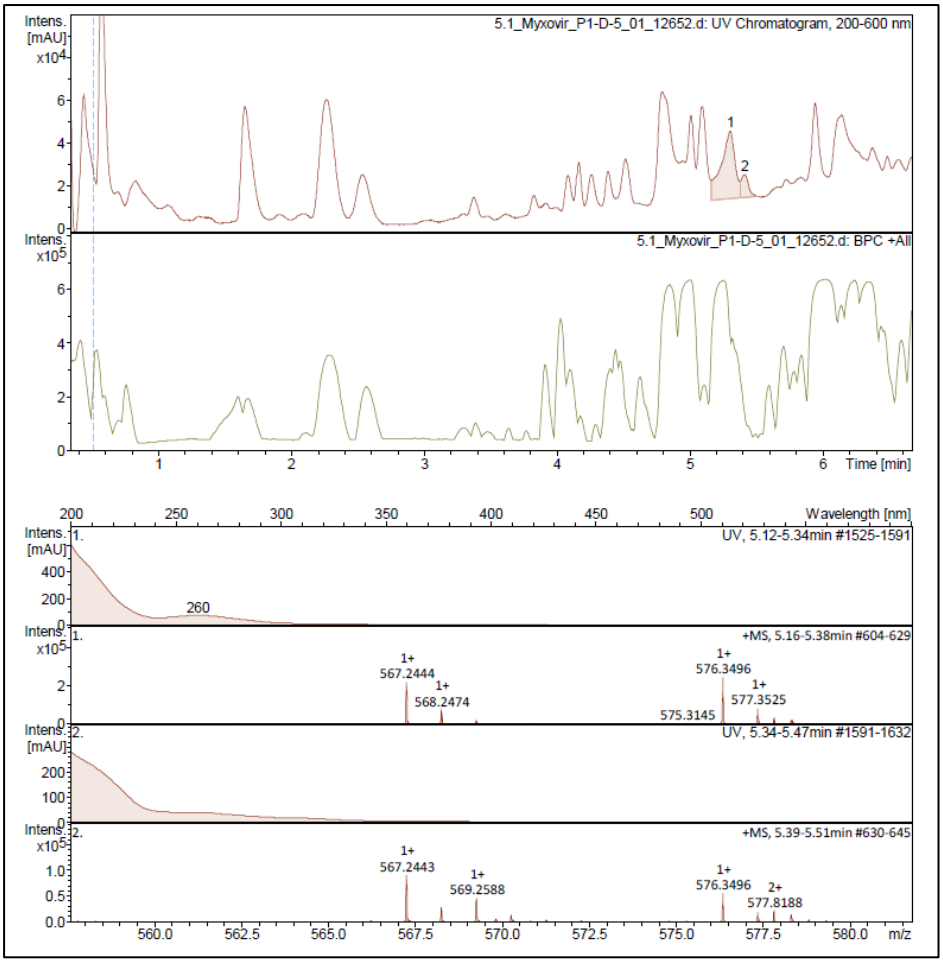


Figure 24: LC-MS chromatogram (UV and mass spectra) of the crude extract of WS 5.1 in Myxovirescin medium. 1: saframycin MX1 BCH; 2: saframycin derivative with typical chlorine isotopic pattern.

3.2. Part 2: Actinobacteria isolated from marine sponges and sediment in Guam: Taxonomic characterization and screening for new bioactive compounds

The marine actinobacterial strains Guam 928, 1257, 1285, 1322, 1509, 1510, 1566, 1582 and 1583 were isolated from marine sponges and sediments in Guam. The strains were screened for the production of secondary metabolites and were taxonomically analyzed.

3.2.1. Phylogenetic analysis

16S rRNA analyses were done for all nine Guam strains (Guam 928, 1257, 1285, 1322, 1509, 1510, 1566, 1582 and 1583). Using the "Blast" tool on the homepage of the "National Centre for Biotechnology Information" (NCBI) (www.ncbi.com) the closest relatives of these strains were detected. The strains were identified as members of the genera *Micromonospora*, *Rhodococcus*, *Microbacterium* as well as *Streptomyces*.

3.2.1.1. Micromonospora

The strains Guam 928, 1509, 1510, 1582 and 1583 were identified as *Micromonospora* sp. strains after 16S rRNA analysis. Because most of these strains had the same relatives (*Micromonospora* type strains with 99% homology in the 16S rRNA sequence) (appendix) all of them were prepared for one phylogenetic tree based on the 16S rRNA sequences as well as for MALDI-TOF analysis as additional tool for the taxonomic differentiation of the strains.

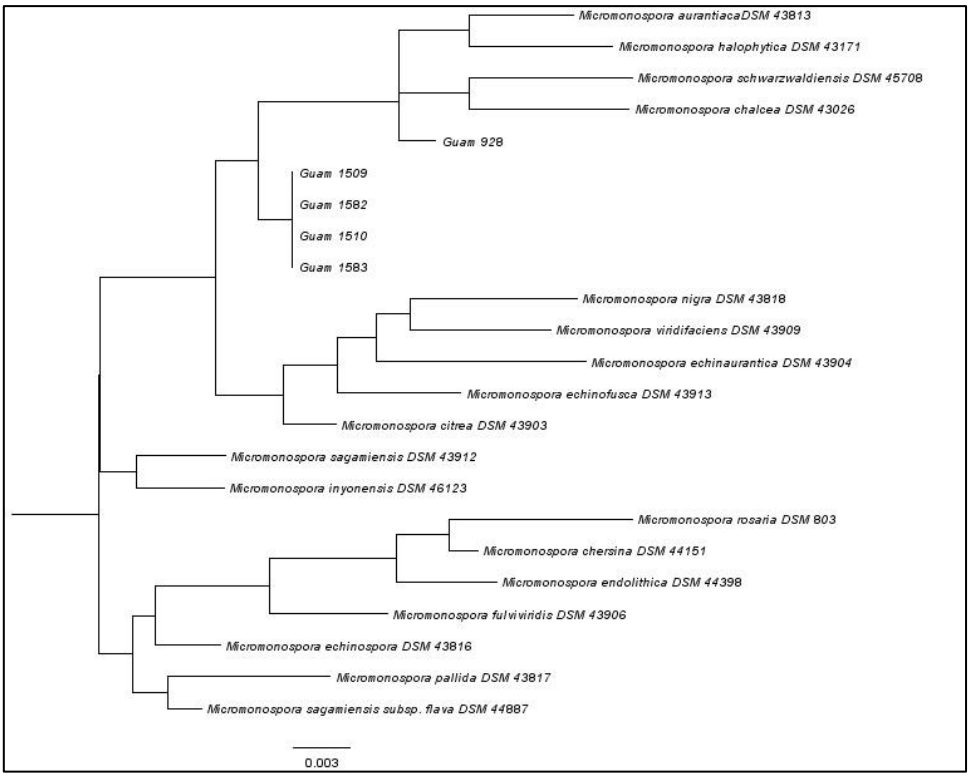


Figure 25: Neighbourhood joining tree of the isolated strains Guam 928, 1509, 1510, 1582 and 1583 and their closest relatives. All *Micromonospora* type strains were detected via "Blast" tool on NCBI and the tree was calculated using GGDC.

Figure 25 shows the phylogenetic tree generated with the GGDC of the DSMZ based on the 16S rRNA sequences. The Guam isolates 1583, 1510, 1509 and 1582 were very closely related and formed a separate cluster, whereas strain Guam 928 clustered together with *M. schwarzwaldiensis* (DSM45708^T), *M. chalcea* (DSM43026^T), *M. halophytica* (DSM43171^T) and *M. aurantiaca* (DSM43813^T).

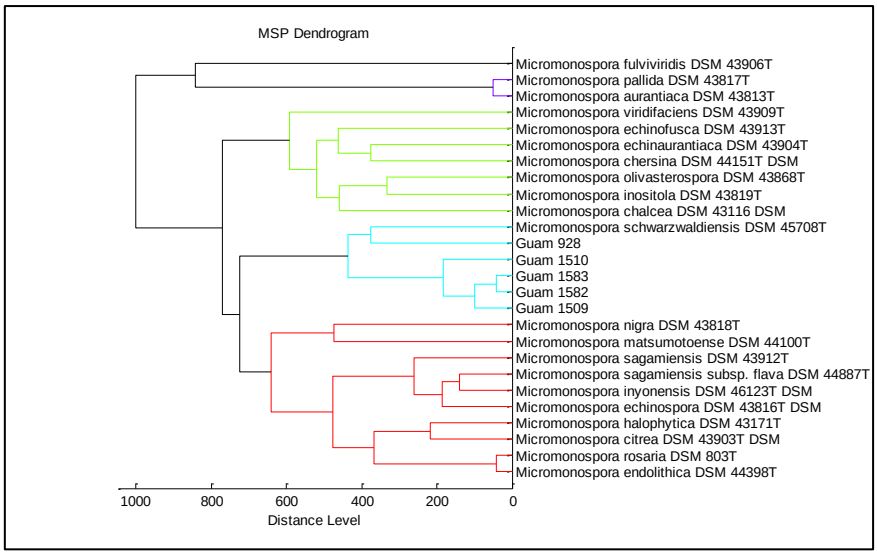


Figure 26: MALDI-TOF dendrogram of the *Micromonospora* strains Guam 928, 1509, 1510, 1582 and 1583 after and their closest relatives.

Similar clustering was observed in the dendrogram of the MALDI-TOF analysis. The strains Guam 1510, 1583, 1582 and 1509 clustered together, however strains Guam 928 and the type strain of *M. schwarzwaldiensis* formed a separate cluster within the Guam cluster. Whereas the other three strains *M. chalcea* (DSM43026^T), *M. halophytica* (DSM43171^T) and *M. aurantiaca* (DSM43813^T) clustered in separate clusters each (Figure 26).

Because of the high amount of very closely related type strains within the 16s rRNA sequence of the isolated Guam strains this study was focused on Guam 928 and 1582 for closer analysis. These two strains showed an interesting secondary metabolite profile. The MALDI-TOF profile of strains Guam 1509, 1510 and 1583 can be found in the appendix.

For a better separation and the more distinct clustering with *M. schwarzwaldiensis*, Guam 928 was analyzed again using MALDI-TOF (Figure 27).

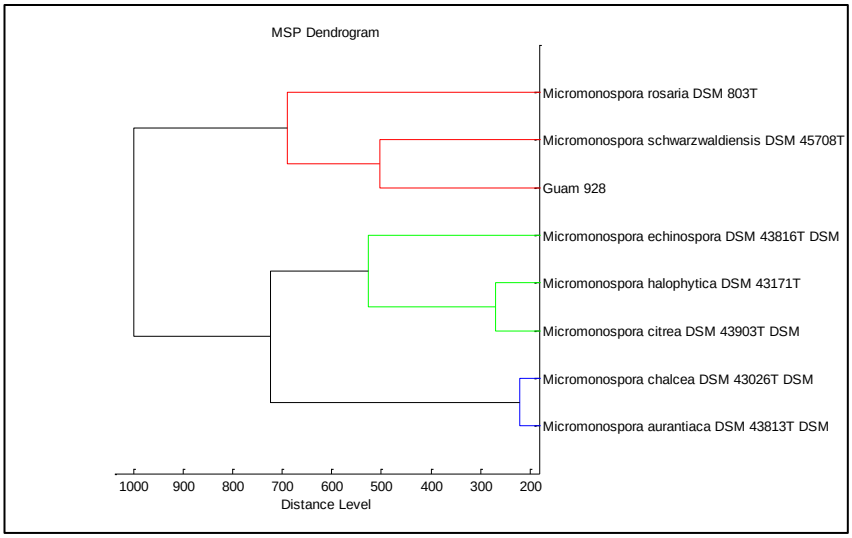


Figure 27: MALDI-TOF dendrogram of the *Micromonospora* strain Guam 928 and its closest relatives.

As detected before, the strain Guam 928 was revealed to be closely related to *M. schwarzwaldiensis* and formed a cluster with *M. rosaria* (Figure 27).

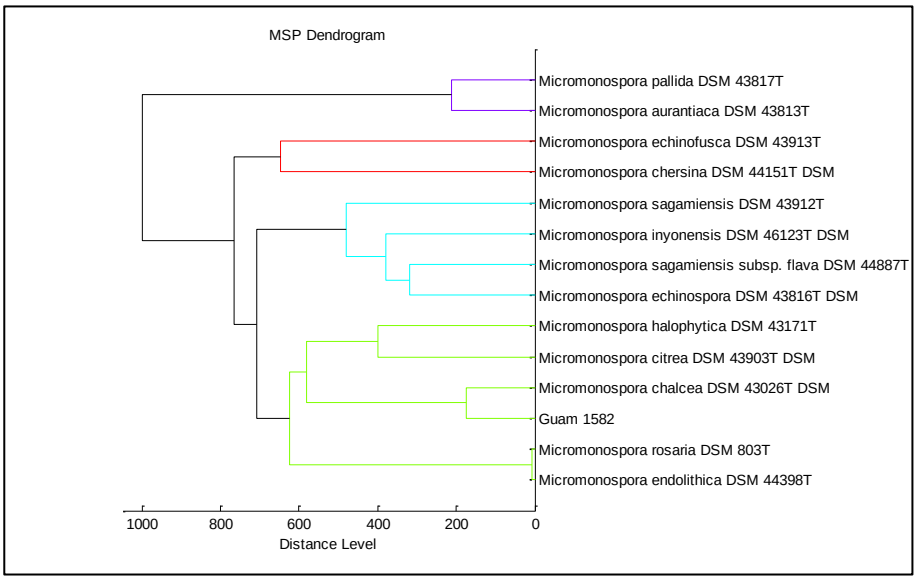


Figure 28: MALDI-TOF dendrogram of the *Micromonospora* strain Guam 1582 and its closest relatives.

MALDI-TOF analysis suggested that the closest relative to strain Guam 1582 was the type strain *M. chalcea* (Figure 28). Because of the interesting and akin secondary metabolite profile of both strains, Guam 1582 was selected for further phylogenetic analysis.

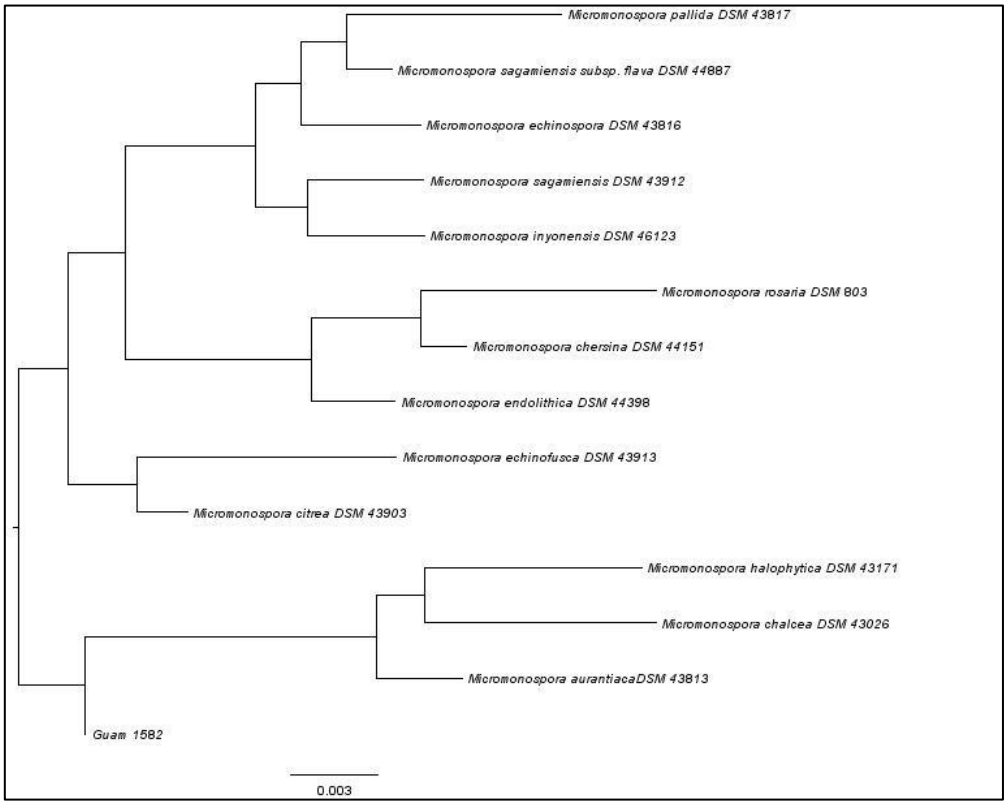


Figure 29: Phylogenetic tree based on the 16S rRNA sequence of Guam 1582 and its closest relatives. All *Micromonospora* type strains were detected via "Blast" tool on NCBI and the tree was calculated using GGDC.

The phylogenetic tree based on the 16S rRNA sequences showed Guam 1582 clustering together with *M. aurantiaca* but also with *M. chalcea* and *M. halophytica* (Figure 29) To be sure that Guam 1582 is not a *M. chalcea* strain, RiboPrinter® analyses were done. These analyses showed that according to the different *PvuII* RiboPrinter patterns (Figure 30) strain Guam 1582 and *M. chalcea* are not identical at strains´ level.

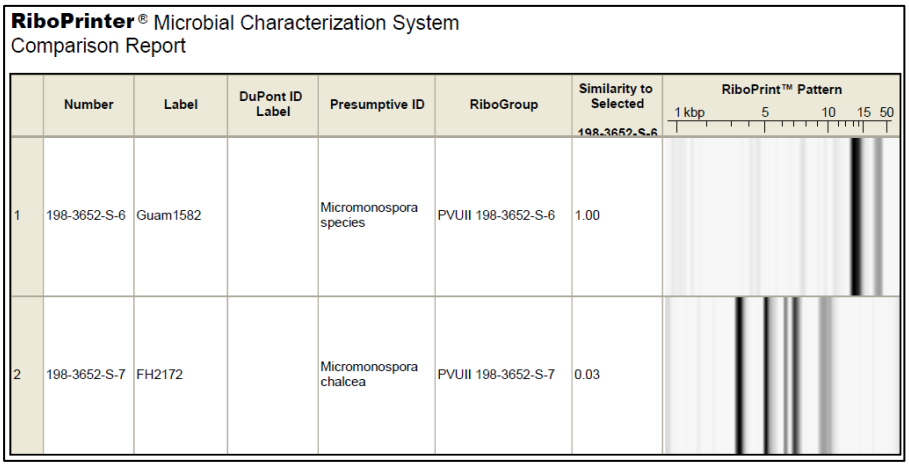


Figure 30: RiboPrinter® results of the *Micromonospora* strain Guam 1582 with its closest relative *M. chalcea*.

3.2.1.2. Rhodococcus

The strain Guam 1566 was at first an interesting strain for the taxonomic description because the closest relatives were the type strain *Rhodococcus corynebacterioides* with 99% and *R. equi* with 97% identity in the 16S rRNA sequence (Table 20). However, because of the high morphological similarity with the type strain *R. corynebacterioides* (data not shown), this strain was skipped for further taxonomic description.

Table 20: Closest relatives (type strains) of the Rhodococcus strain Guam 1566 after using the "Blast" tool on NCBI. Based on the homology of the 16S rRNA sequence

Guam 1566	
R. corynebacterioides (99%)	DSM 20151^T
R. equi (97%)	DSM 20307^T

3.2.1.3. Microbacterium

The strain Guam 1257 showed 97% identity in the 16S rRNA sequence (Table 21) with 8 strains, revealing it as a possibly new strain. However, no interesting metabolite profile was detected and therefore no further taxonomic analysis was done in this study.

Table 21: Closest relatives (type strains) of the *Microbacterium* strain Guam 1257 after using the "Blast" tool on NCBI. . Based on the homology of the 16S rRNA sequence.

Guam 1257	
M. testaceum (97%)	DSM 20166^T
M. chocolatum (97%)	DSM 12507^T
M. esteraromaticum (97%)	DSM 8609^T
M. arabinogalactanolyticum (97%)	DSM 8611^T
M. kitamiensis (97%)	DSM 13237^T
M. trichothecenolyticum (97%)	DSM 8608^T
M. thalassium (97%)	DSM 12511^T
M. hominis (97%)	DSM 12509^T

3.2.1.4. Streptomyces

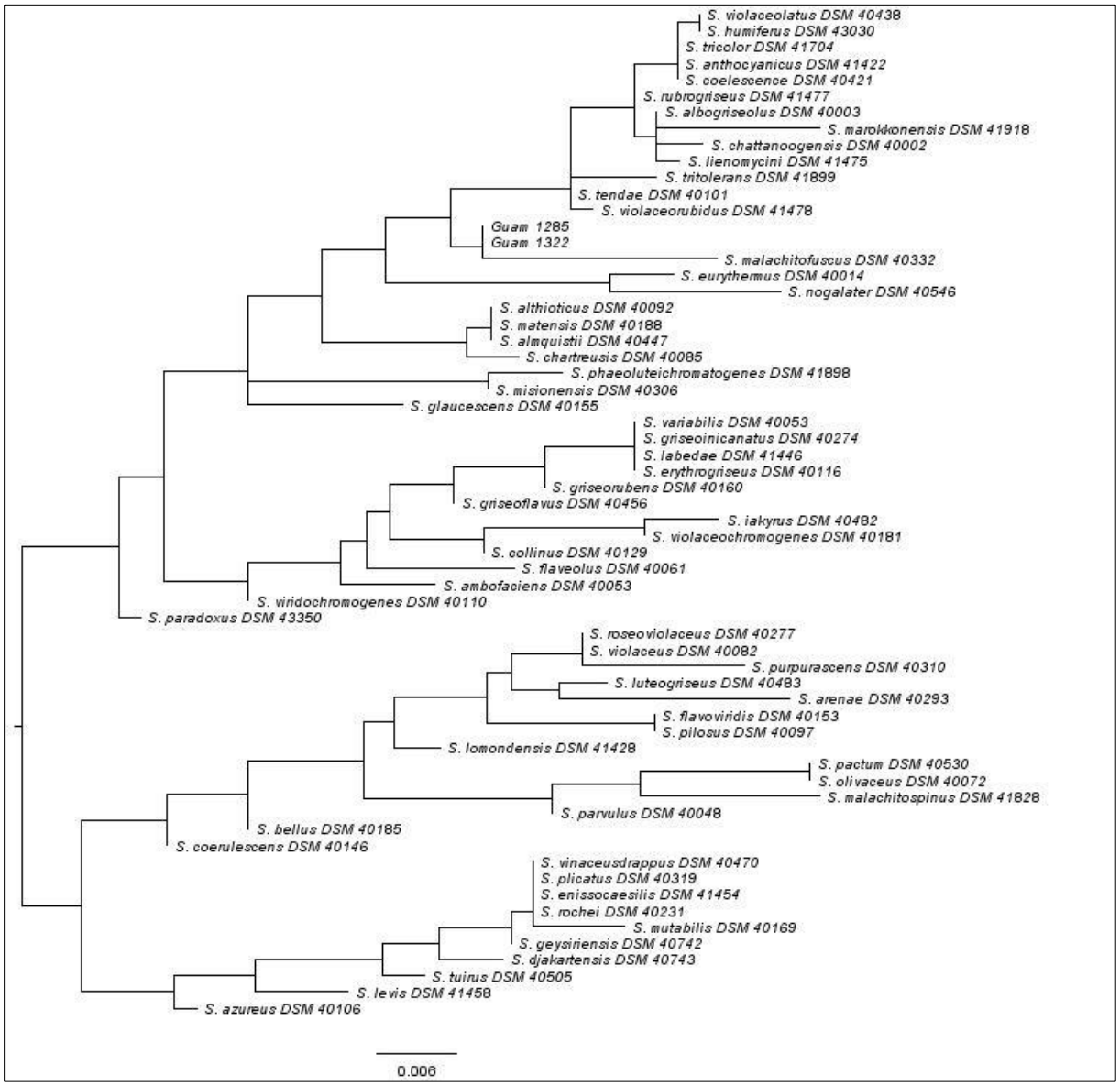


Figure 31: Phylogenetic tree based on the 16S rRNA sequence of the isolated strains Guam 1285 and 1322 and their closest relatives. All *Streptomyces* type strains were detected via "Blast" tool on NCBI and the tree was calculated using GGDC.

The genus *Streptomyces* is one of the largest bacterial groups with most members being plylogenetically very closely related. Hence, being 99% identical in the 16S rRNA sequence with 61 other *Streptomyces* type strains was not surprising for the two isolated *Streptomyces* strains Guam 1285 and Guam 1322 (Figure 31). Because of the high number of related type strains it was decided to stop working on the taxonomic description of the *Streptomyces* strains Guam 1322 and 1285.

3.2.2. Secondary metabolites produced by marine actinobacteria from Guam

All nine Guam strains were screened in two standard media used for the screening for secondary metabolites (5254 and 5294 medium). Additionally, because of the marine origin of the isolates, the strains were screened on the same media prepared with artificial sea water. After cultivation and preparation of crude extracts, the bioactivity was tested for antimicrobial effects in the serial dilution assay. Interesting compounds were detected by fractionation experiments and identified using LS-MS analysis. The production rate of probably new compounds was increased by media optimization, kinetics and "upscaling" experiments to finally isolate and purify the substance for structure elucidation.

3.2.2.1. Results of bioactivity tests: serial dilution assay of the standard and multi resistant test panel

After the cultivation of strains Guam 928, 1257, 1285, 1322, 1509, 1510, 1566, 1582 and 1583 in the different screening media crude extracts were prepared. Each extract was screened on the standard test panel. Extracts showing strong activities were tested on the multi resistant test panel.

Most of the extracts produced by the Guam strains were detected to strongly inhibit the growth of Gram-positive bacteria from the standard test panel, however some of the strains also produced compounds which show higher bioactivity against Gram-negative bacteria, e. g. *C. violaceum E. coli* and *E. coli* TolC (appendix). Because of the high number of growth inhibitions the most interesting strains Guam 928, Guam 1257, Guam 1285, Guam 1509, Guam 1510, Guam 1566 and Guam 1582, were additionally tested on the multi resistant test panel (Table 22).

Table 22: MIC of the extracts from the isolated actinobacteria from sponges and marine sediment in Guam against the multi resistant test panel in different media: [4] 5254 medium; [5] 5254 sea water medium; [6] 5294 medium; [7] 5294 sea water medium.

	Fungi	**Gram +**				**Gram -**				
	C. albicans (DSM 1665)	*S. aureus* Newman*	*S. aureus* N315 (DSM 11822)	*E. faecium* (DSM 20477)	*E. faecium* (DSM 17050)	*P. aeruginosa* PA14 (DSM 19882)	*E.coli* (DSM 1116)	*E.coli* WT-3	*E.coli* XL-1 blue	*E.coli* ESBL (DSM 22664)
Guam 928[4]	-	-	-	-	A	-	-	-	-	A
Guam 928[5]	-	D	D	C	A	-	-	-	-	-
Guam 1257[4]	-	B	-	-	A	-	-	-	-	-
Guam 1257[5]	-	B	-	-	-	-	-	-	-	-
Guam 1285[4]	-	H	H	H	-	-	D	E	G	-
Guam 1285[5]	-	H	H	H	H	B	C	D	E	-
Guam 1322[4]	-	H	H	H	-	-	A	D	F	E
Guam1322[5]	-	H	H	H	H	-	D	D	E	-
Guam 1509[4]	-	B	-	-	A	-	-	-	F	-
Guam1510[4]	-	-	-	-	-	B	-	-	-	-
Guam 1566[4]	-	E	D	-	-	-	-	-	A	-
Guam1582[6]	-	D	A	A	C	-	-	-	A	-
Guam 1582[7]	-	D	D	E	F	-	-	-	B	-

Most of the extracts generated a high number of growth inhibitions in the standard as well as in the multi resistant test panel. Therefore, a few extracts were chosen for further fractionation experiments to correlate the biological activity to the corresponding peaks in the HPLC chromatogram.

3.2.2.2. Interesting compounds produced by marine actinobacteria from Guam

Most of the bioactivity, determined with the serial dilution assay could not be reproduced in the fractionation experiments. Furthermore, in some cases, the amount of produced bioactive compound was too low or the ionization was very poor. Therefore only strains Guam 1582 and 928 were chosen for further metabolite analysis.

3.2.2.2.1. Strain Guam 1582: producer of rakicidin A, B and E

The raw extract of Guam 1582 showed a strong activity against the VREF and MRSA strains. Hence, the extract was fractionated to localise the retention time and detect the biologically active compound.

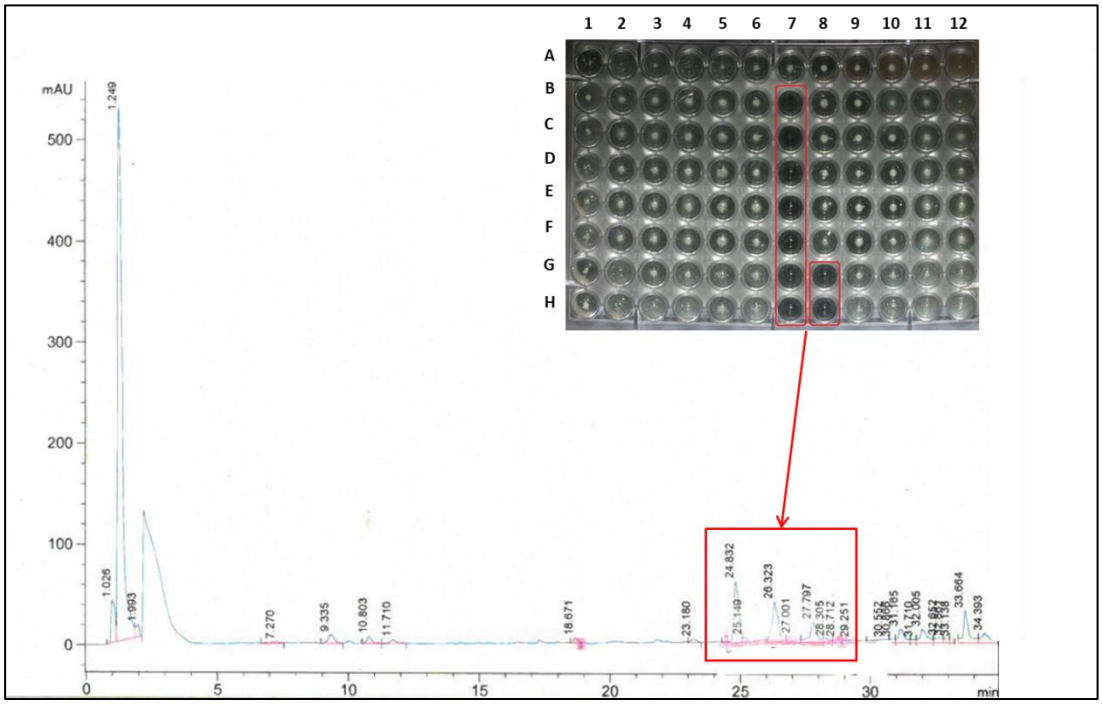


Figure 32: Correlation between the bioactivity of the extract of Guam 1582 in 5294-SW medium against the test organism *E. faecium* DSM 17050 (VREF) and the corresponding area in the HPLC chromatogram.

Due to the fractionation three peaks could be correlated to the biological activity, with retention times between 24 and 28.5 min (Figure 32).

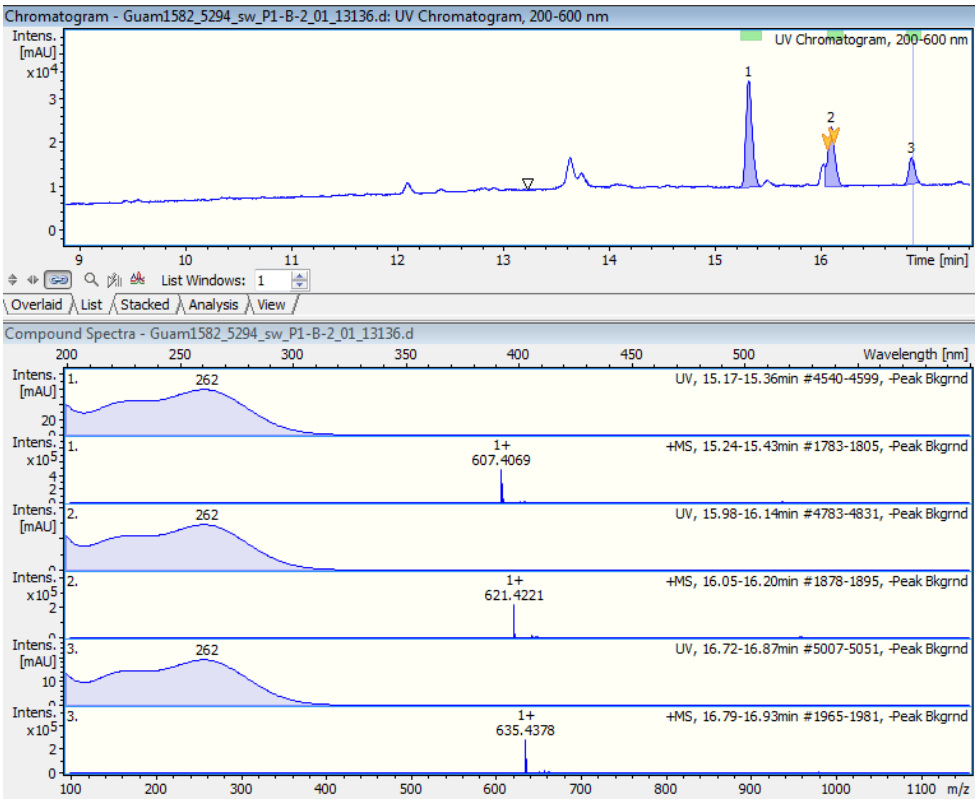


Figure 33: LC-MS chromatogram (UV and mass spectra) of the extract of Guam 1582 in 5294-SW medium. The three peaks were correlated to the masses 1: m/z 607.4069 $[M+H]^+$ (t_R = 24.02 min); 2: m/z 621.4221 $[M+H]^+$ (t_R = 26.4 min); 3: m/z 635.4378 $[M+H]^+$ (t_R = 27.0 min).

Comparing the HPLC chromatogram from the fractionation with the LC-MS chromatogram the three peaks were identified using the characteristic UV chromophores, revealing the masses to be *m/z* 607.4069 $[M+H]^+$ (t_R = 24.02 min), *m/z* 621.4221 $[M+H]^+$ (t_R = 26.4 min) and *m/z* 635.4378 $[M+H]^+$ (t_R = 27.0 min), respectively, indicating structurally related (Figure 33).

To detect the best conditions for the production of the three bioactive compounds, the strain Guam 1582 was cultured in medium 5294 prepared with dest. water or artificial sea water as well as different peptones. Furthermore, a fresh reactivated and a strain which was inoculated several times, were compared.

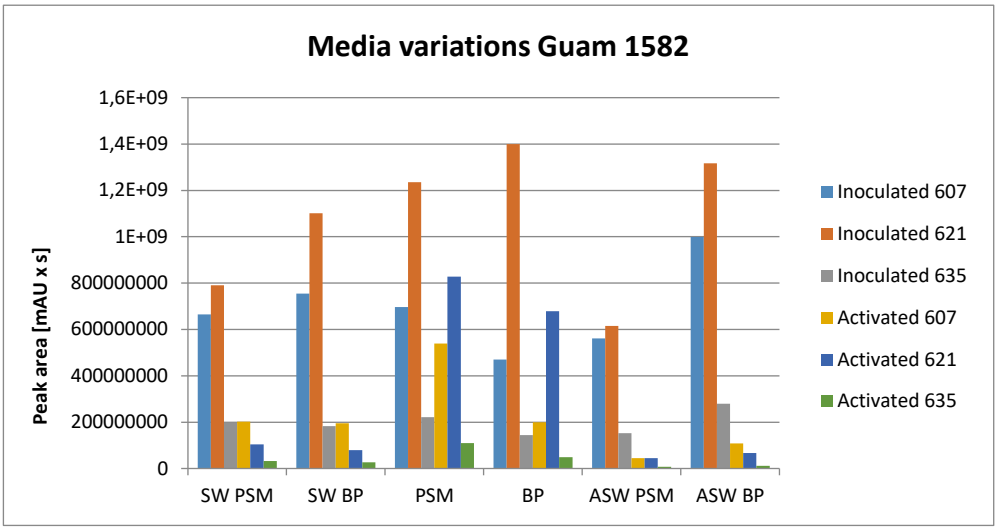


Figure 34: Media variation of Guam 1582. Production of the compounds with the masses 607.4069, 621.4221 and 635.4378 $[M+H]^+$ by the strain Guam 1582 in different media: SW PMS: 5294 medium with sampled sea water and soya peptone Macor; SW BP: 5294 medium with sampled sea water and Bacto peptone; PSM: 5294 medium with dest. water and soya peptone Marcor; BP: 5294 medium with dest. water and Bacto peptone; ASW PSM: 5294 medium with artificial sea water and soya peptone Marcor; ASW BP: 5294 medium with artificial sea water and Bacto peptone.

All three compounds were produced in every medium combination. The best producer strain was the one which was inoculated several times before it was used as preculture. It could be clearly seen that the compound with the molecular cluster at *m/z* 621.4221 $[M+H]^+$ was produced in the highest amounts followed by the compounds with the molecular clusters at *m/z* 607.4069 and 635.4378 $[M+H]^+$. However the medium resulting in the best production for all three compounds was the 5294 medium with artificial sea water and Bacto peptone (ASW BP) (Figure 34).

To find the best day to harvest the strain Guam 1582 to get the highest amount of the three wanted compounds, six 250 mL flasks filled with 100 mL were inoculated 1:10 with a 5 day old preculture of the strain Guam 1582. From day 3 to 8 after inoculation, every day one flask was harvested and analyzed.

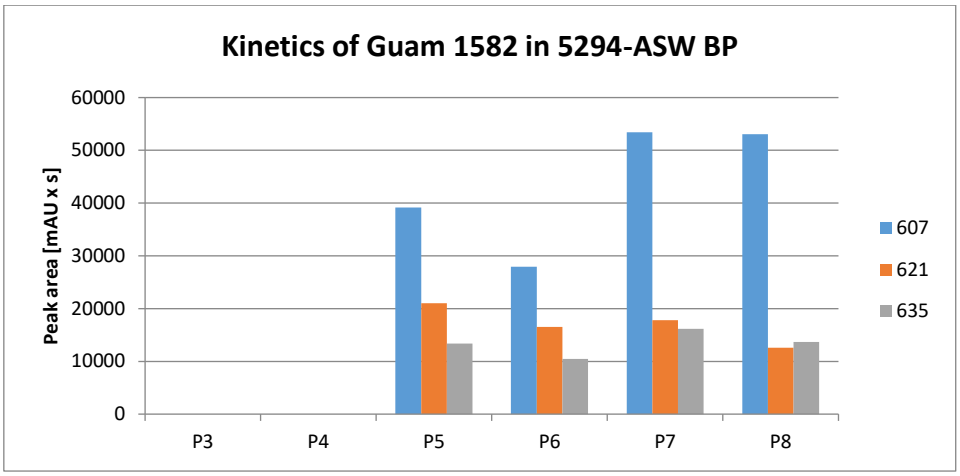


Figure 35: Kinetics of Guam 1582 in 5294-ASW BP. Production of the compounds with the masses 607.4069, 621.4221 and 635.4378 $[M+H]^+$ by the strain Guam 1582 in 5294 medium with artificial sea water and bacto peptone (5294-ASW BP). The production of these compounds was detected by the harvest of one flask from day 3-8 (P3-P8) and following LCMS analysis.

All three compounds were produced from day 5 to 8. Finally, day 7 showed the highest production of all three compounds und was therefore chosen as best day for harvesting (Figure 35).

For the "upscaling" experiments 50 250 mL flasks filled with 100 mL 5294-ASW medium were inoculated 1:10 with a 5 day old preculture of a well grown strain. Afterward the cultures were incubated at 30 °C and 160 rpm for 7 days.

The isolation, purification and structure elucidation was done by Sabrina Karwehl (PhD student MWIS, HZI Braunschweig).

The compound with the molecular cluster $[M+H]^+$ at *m/z* 607.4069 was identified as the known antibiotic Rakicidin A ($C_{32}H_{54}N_4O_7$), the compound with the molecular cluster $[M+H]^+$ *m/z* 621.4221 as Rakicidin B ($C_{33}H_{56}N_4O_7$) and the compound with molecular cluster $[M+H]^+$ *m/z* 635.4378 as Rakicidin E ($C_{34}H_{58}N_4O_7$) (Figure 36).

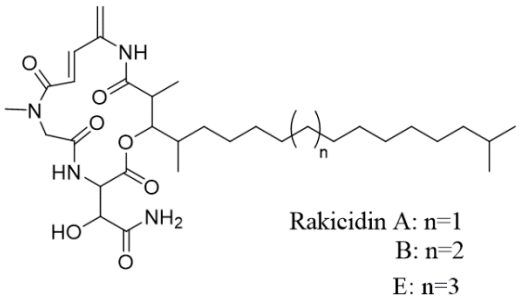


Figure 36: Chemical structures of the compounds rakicidin A, B and E.

To test the bioactivity of the pure compounds of rakicidin A, B and E, a bioactivity test (serial dilution assay) was done against the MRSA strain *Staphylococcus aureus* N315 and VREF strain *Enterococcus faecium* DSM-17050 in different concentrations.

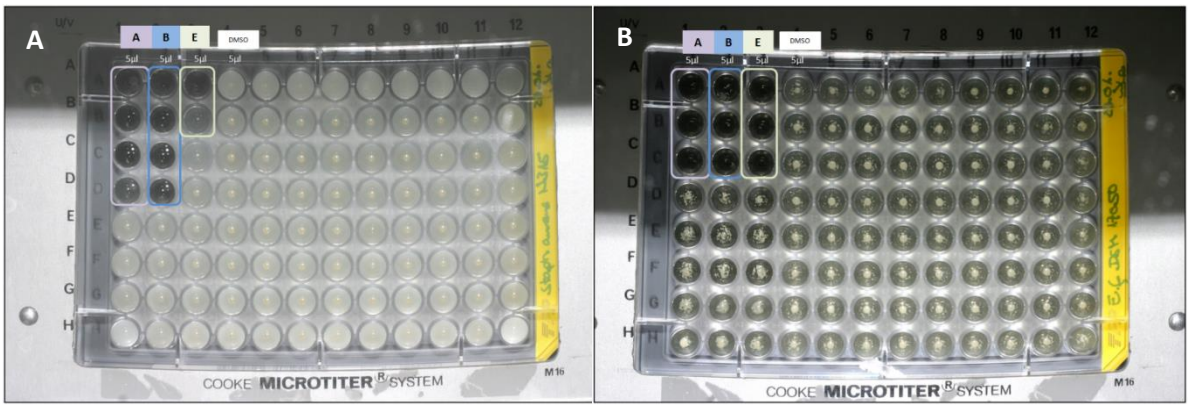


Figure 37: Results of serial dilution assays of Rakicidin A, B and E. 5 µL of a 1 mg/mL in DSMO concentrated stock solution was inserted into the assay. The solvent DSMSO was used as negative control. A: serial dilution assay against *Staphylococcus aureus* N315 (MRSA strain); B: serial dilution assay against *Enterococcus faecium* DSM-17050 (VREF).

Rakicidins A, B and E showed selective antibiotic activities against Gram-positive bacteria, including the methicillin resistant *Staphylococcus aureus* N315 (MRSA) and the vancomycin resistant *Enterococcus faecium* DSM-17050 (VREF) (Figure 37) as well as an inhibition of the murine fibroblast cell line L929. The biological activities against *Micrococcus luteus* (1.0 µg/mL), *Bacillus subtilis* (4.2 µg/mL) and the VREF strain (4.2 µg/mL) were detected for all tested rakicidins at a similar concentration. In contrast, against *S. aureus* rakicidin A showed the lowest MIC value of 2.1 µg/mL and rakicidins B and E higher values of 4.1 µg/mL and 8.3 µg/mL, respectively. All rakicidins had the same MIC values (4.1 µg/mL) against the MRSA strain but against the VREF strain rakicidin E showed considerably decreased activity with a MIC value of 16.5 µg/mL compared to 2.1 µg/mL for rakicidin A and B (Table 23).

Table 23: MIC [in µg/mL] values of rakicidins A, B and E Gram-positive and -negative bacteria (including multi resistant strains), fungi as well as the murine cell line L929.

	Test organism	Rakicidin A	Rakicidin B	Rakicidin E
Fungi	*Schizosaccharomyces pombe* (DSM70572)	-	-	-
	Pichia anomala (DSM6766)	-	-	-
	Candida albicans (DSM1665)	-	-	-
	Mucor hiemalis (DSM2656)	-	-	-
	Rhodosporidium toruloides (DSM10134)	-	-	-
Gram$^+$	*Micrococcus luteus* (DSM1790)	1.04	1.04	1.04
	Bacillus subtilis (DSM10)	4.16	4.16	4.16
	Staphylococcus aureus Newman*	2.07	4.14	8.29
	Staphylococcus aureus N315 (DSM11822) (MRSA)	2.07	2.07	16.50
	Enterococcus faecium (DSM20477)	4.14	4.14	8.29
	Enterococcus faecium (DSM17050) (VREF)	4.14	4.14	4.14
	Mycobacterium sp. (DSM43270)	-	-	-
Gram$^-$	*Chromobacterium violaceum* (DSM30191)	-	-	-
	Pseudomonas aeruginosa (DSM19882)	-	-	-
	Escherichia coli (DSM1116)	-	-	-
	Escherichia coli WT-3 (quinolone resistant)	-	-	-
	Escherichia coli XL-1 blue (tetracycline resistant)	-	-	-
	Escherichia coli ESBL (DSM-22664)	-	-	-
	Murine cell line L929	1.22	1.22	1.22

3.2.2.2.2. Strain Guam 928: producer of aloesaponarin II and 5-hydroxyaloesaponarin II

The crude extract of Guam 928 showed a strong growth inhibition against the MRSA strain *Staphylococcus aureus* N315 and also a slight inhibition against the VREF strain *Enterococcus faecium* DSM 17050. Thus, it was decided to further analyse this extract using semi-preparative HPLC fractionation as already described above for other strains.

Both biological activities of the crude extract were correlated to compound 2 (K2) (see Figure 38 and 39). Furthermore, a second area of inhibition was revealed on the fractionation plate inoculated with VREF. Unfortunately this area was too broad with no characteristic peaks (compound 3/K3), resulting in the dismissal of this area for further investigations during this study. Despite its biological inactivity, a third peak (compound 1/K1) was revealed due to its close proximity to compound 2, height and its UV chromophore being similar to that of compound 2.

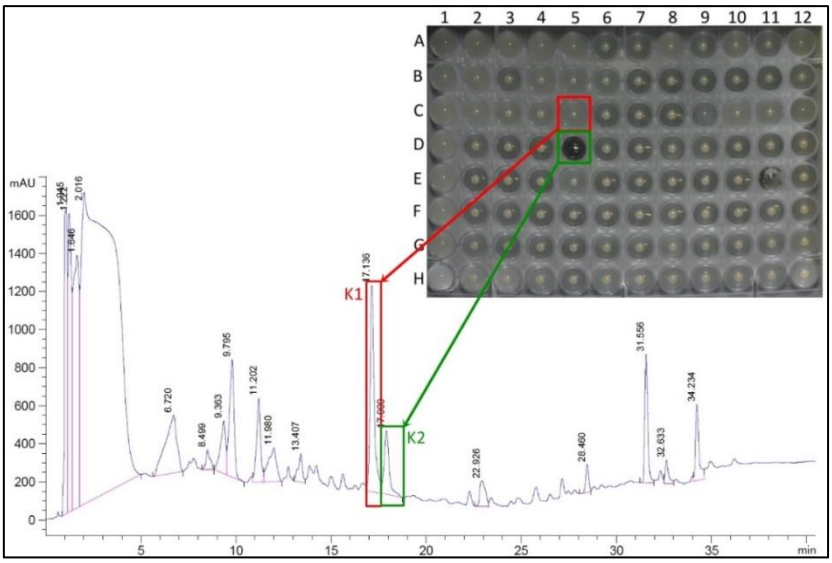


Figure 38: Correlation of the biological activity of the extract of Guam 928 in 5294 medium against the test organism *S. aureus* N315 (MRSA) and the corresponding HPLC chromatogram. K1: compound 1, K2: compound 2.

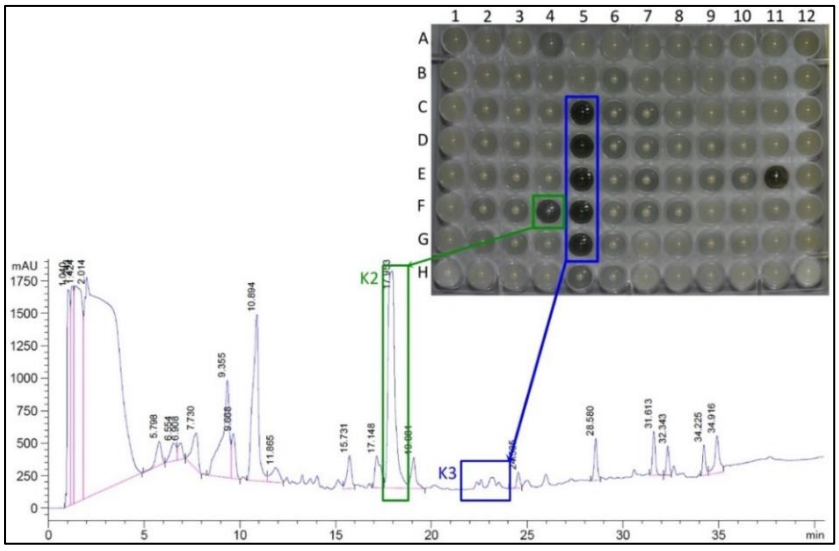


Figure 39: Correlation of the biological activity of the extract of Guam 928 in 5330 medium against the test organism *E. faecium* DSM 17050) and the corresponding HPLC chromatogram. K2: Compound 2, K3: Compound 3.

The LC-MS analyses showed of the molecular cluster $[M+H]^+$ at *m/z* 255.0647 (t_R = 10.9 min) for compound 1 (K1) and the molecular cluster $[M+H]^+$ at *m/z* 271.0596 (t_R = 11.4 min) for compound 2 (K2) (Figure 40).

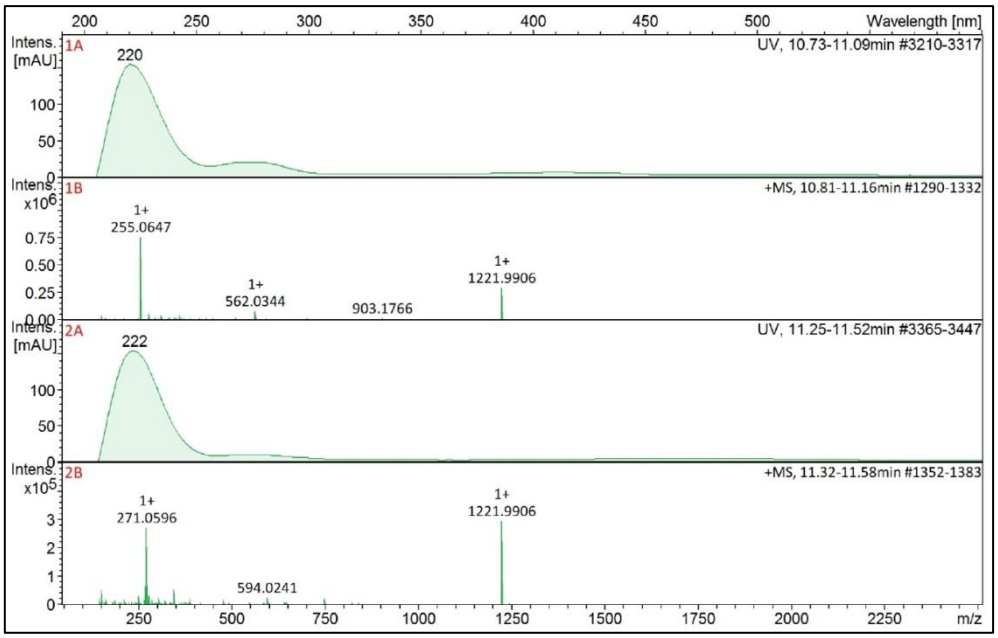


Figure 40: LC-MS run of the crude extract of Guam 928 in 5294 medium. UV absorption (1A und 2A) und mass spectra (1B und 2B) of compound 1 (K1) and compound 2 (K2).

The media variation revealed medium 5294 as the best production medium for compounds 1 (K1) and 2 (K2) (Figure 41A). To enhance the yield of the compounds, the composition of medium 5294 was varied using dest. water (dW), sampled sea water of the coast of Neuharlingersiel (SW) and artificial sea water (ASW) and was harvested at day 5 (T5) and 7 (T7) after inoculation. This variation and kinetic experiments revealed the best medium composition to be the one using artificial sea water (ASW) and the harvest point at day 7 (Figure 41B).

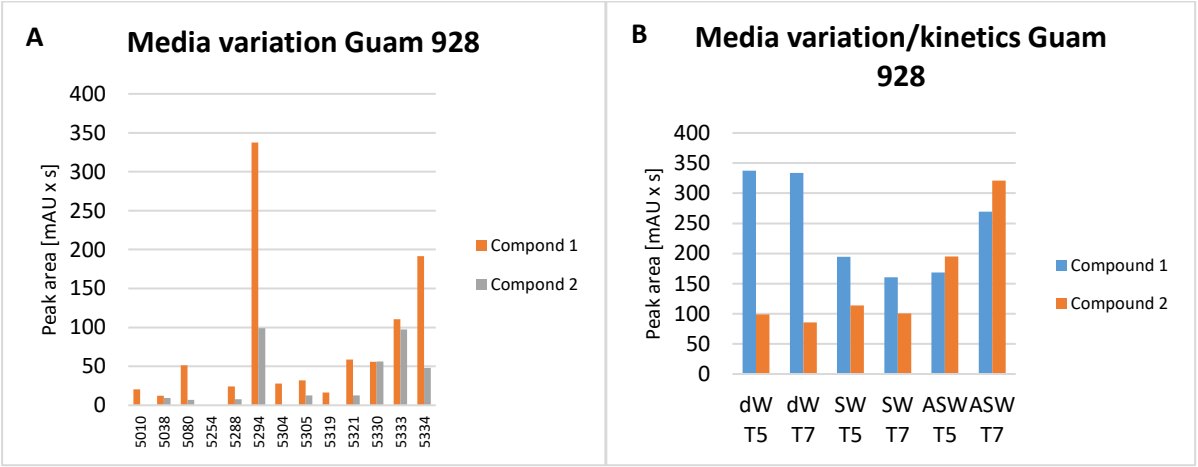


Figure 41: Media variation and kinetics of Guam 928. To detect the production of the compounds 1 (K1) and 2 (K2) with the masses of 255.0647 and 271.0596 $[M+H]^+$m/z, the peak area was determined. A: For amplified media variation of the strain Guam 928 was cultured in the media: 5010, 5038, 5080, 5254, 5288, 5294, 5304, 5305, 5329, 5321, 5330, 5333 and 5334.B: The media variation and kinetics of the strain Guam 928 was done in the screening medium for actinobacteria 5294 with dest. water (dW), sampled sea water of the coast of Neuharlingersiel (SW) and artificial sea water (ASW) and was harvested at day 5 (T5) and 7 (T7) after inoculation.

Unfortunately, the yield of the compounds was too low for compound isolation so further media composition variations of the medium 5294 were done. Therefore, a total variation of medium 5294 was done. Each component of the medium was left out in one approach and additionally to the variation of the water (dest. and artificial sea water) and the variation of the age of the preculture (fresh or several times inoculated; in 5294 or GYM medium), different components out of the marine environment like shells and chitin (Table 24) were added.

The variation of medium 5294 for the production of the compounds 1 (K1) and 2 (K2) showed high variation. The different precultures as well as diverse media compositions caused a very high discrepancy between the approaches. The medium 5294-ASW with the addition of chitin and the fresh inoculated preculture in GYM medium showed the highest production of the compounds K1 and K2 (Figure 42).

Table 24: Media compositions of 5294 medium for the production enhancement of the compounds 1 (K1) and 2 (K2) of the strain Guam 928.

	Media compositions of 5294 medium
1	5294 medium ASW
2	5294 medium ASW without starch
3	5294 medium ASW without yeast extract
4	5294 medium ASW without glucose
5	5294 medium ASW without corn steep
6	5294 medium ASW without peptone
7	5294 medium
8	5294 medium with seashells (10g/l)
9	5294 medium with algea (1g/L)
10	5294 medium with seashells (10g/l) and algae (1g/l)
11	5294 medium with chitin (1g/l)
12	5294 medium with sea sand (1g/l)
13	5294 medium with NaBr (10g/l)
14	5294 medium ASW without glycerol
15	5294 medium with cobalt chloride (1g/l)
16	5330 medium

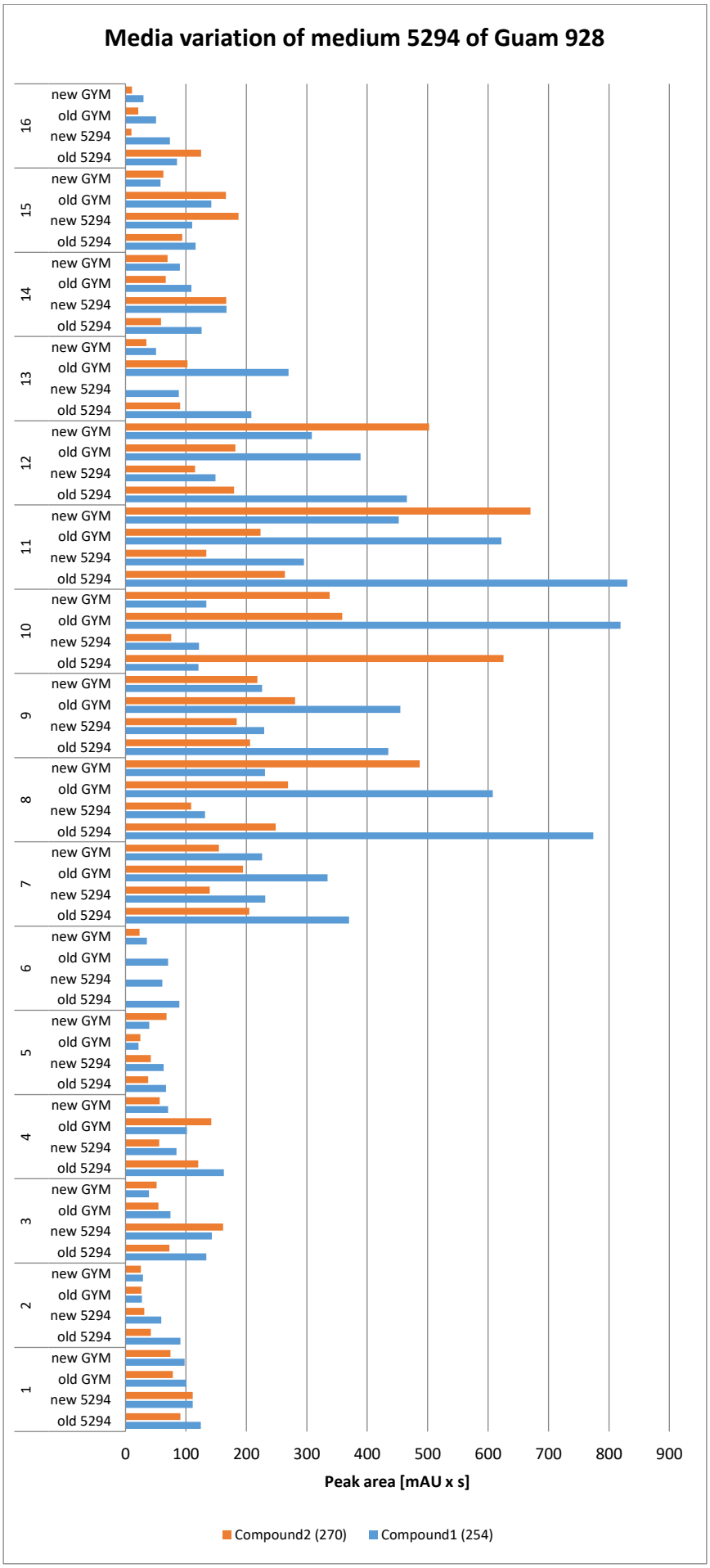


Figure 42: Media variation of medium 5294 of Guam 928. To detect the production of the compounds 1 (K1) and 2 (K2) with the masses of 255.0647 and 271.0596 $[M+H]^+$m/z, the peak area was determined. The composition of the medium 5294 was variated with marine ingredients. The media compositions 1-16 can be taken out of table 24. Every medium composition was inoculated with a preculture a fresh inoculated preculture or a preculture with was inoculated several times before. Furthermore, the precultures were cultured in GYM and 5294 Medium.

For the “upscaling” experiments 50 250 mL flasks filled with 100 mL 5294 medium with artificial sea water and chitin were inoculated 1:10 with a 5 day old preculture of a well grown strain in GYM medium. Afterward the cultures were incubated at 30 °C and 160 rpm for 7 days.

The purification and structure elucidation were done by Sabrina Karwehl (HZI Braunschweig). After the NMR analysis, both masses were correlated to known compounds. With a molecular mass of 254 Da the inactive compound was identified as Aloesaponarin II ($C_{15}H_{10}O_4$) (Bartel et al. 1990) and the active compound, with a molecular mass of 270 Da, as its derivative: 5-hydroxyaloesaponarin ($C_{15}H_{10}O_5$) (Bystrykh et al. 1997) (Figure 43).

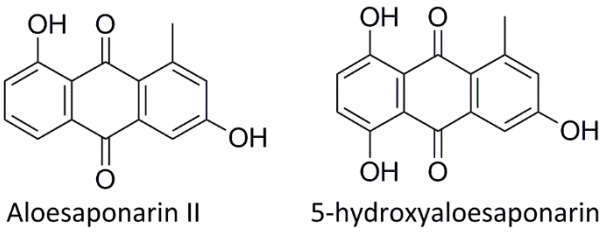


Figure 43: Chemical structures of aloesaponarin II and 5-hydroxyaloesaponarin II.

3.3. Part 3: Actinobacteria isolated from marine and rhizosphere sediment from mangroves in India: Taxonomic characterization and screening for new bioactive compounds

The marine actinobacteria ICN4, 16, 18, 19, 21, 26, 27, 28 and 32 were isolated from marine and rhizosphere sediments from mangrove plants in Tamil Nadu, India. The strains were screened for the production of secondary metablites and were taxonomically analyzed.

3.3.1. Characterization

3.3.1.1. Phylogenetic analysis

ICN16, 18, 19, 21, 26 and 27 were identified as members of the genus *Streptomyces* using the "Blast" tool on the homepage of the NCBI after sequencing the 16S rRNA. As explained above, this phylogenetic group is huge and its members are closely related to each other. For this reason it is not remarkable that the 16S rRNA sequence of ICN16 is 99% identical to 55, ICN18 to 58, ICN26 to 52 and ICN27 to 53 *Streptomyces* type strains. However, most of the closely related type strains are identical to each other and therefore one phylogenetic tree was calculated from all type strains which show 99% identity to the 16S rRNA sequence of the 8 ICN strains (Figure 44). Moreover, strains ICN19 and 21 were detected to be new species. However, both strains show a 100% identity on 16S rRNA to each other as well as 99% with the type strain of *Streptomyces wuyanensis* (DSM 42132^T). Therefore, further studies focused on strains ICN19 and 21.

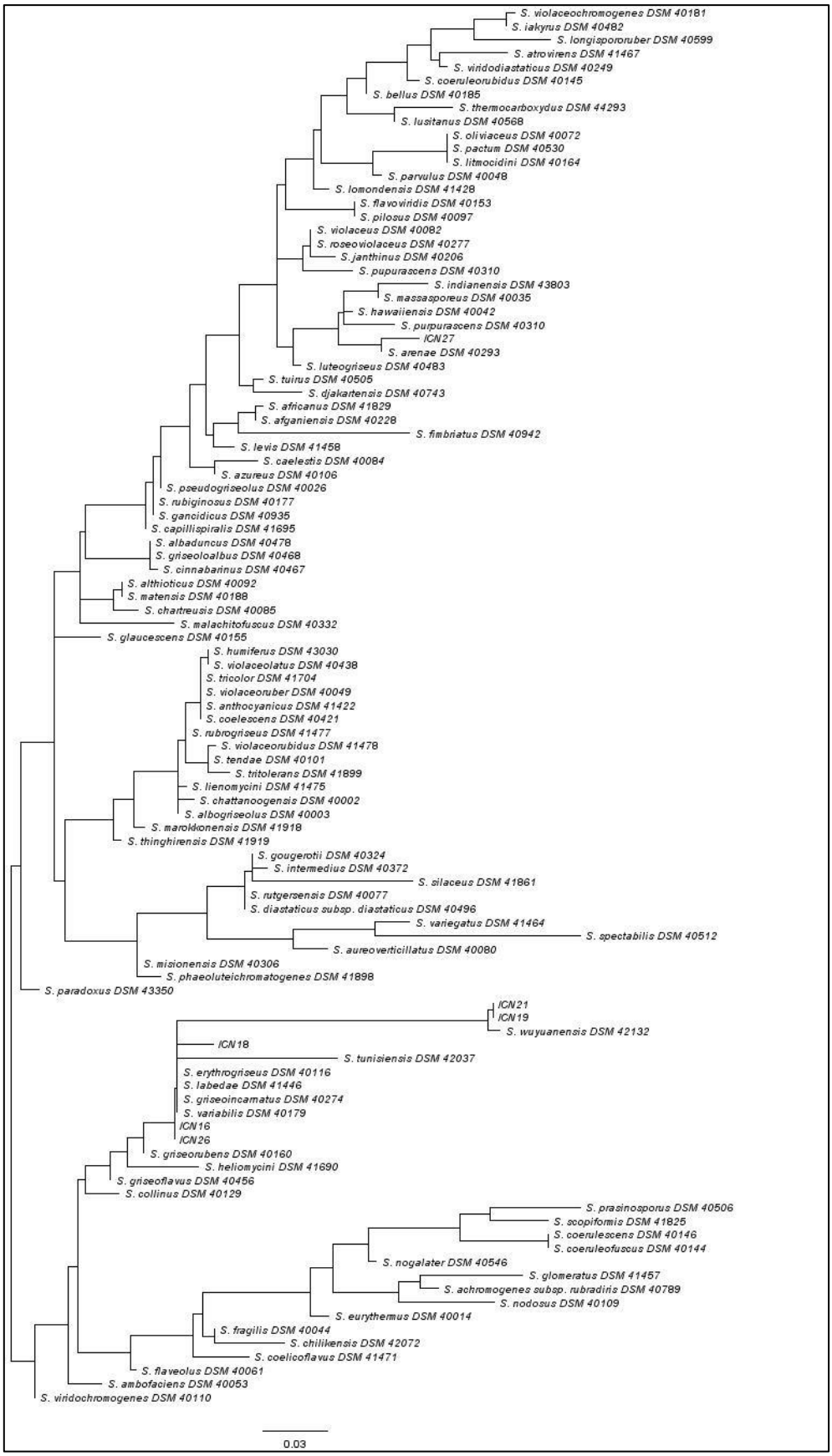


Figure 44: Phylogenetic tree based on the 16S rRNA sequence of strains ICN16, 18, 19, 21, 26 and 27 and their closest related type strains. All type strains were detected via "Blast" tool on the NCBI homepage and have more than 99% homology in the 16S rRNA sequence to the ICN strains. All *Streptomyces* type strains were detected via "Blast" tool on NCBI and the tree was calculated using GGDC.

The strain ICN32 was highly contaminated and the DNA of strain ICN28 could not be isolated in a high range. Therefore these two strains were not used for phylogenetic analysis.

3.3.1.2. Morphology and physiology of strains ICN19 and ICN21

Using 16S rRNA analysis, the two strains ICN19 and 21 were identified to be the same species, however, morphologically, these two strains show some differences. While ICN19 grew well on every ISP medium besides ISP7, ICN21 only grew well on ISP3. Furthermore, ICN21 merely produced areal mycelium on ISP3. In contrast, ICN19 as well as its closest relative, *Streptomyces wuyuanensis* formed grey/white areal mycelia on every ISP medium except ISP6 and 7. On ISP4, 5, 6 and 7 both strains had a beige/ivory colour but on ISP2 ICN19 had a reddish while ICN21 a yellow colony colour as well as on ISP3 on which ICN19 colonies were pink and ICN21 colonies blue. Soluble pigments were not produced by any of the three strains (Table 25).

Table 25: Comparison of the morphology (growth, colony colour, aerial mycelium and soluble pigments on ISP plates) of the strains ICN19, 21 and the closest related type strain *S. wuyuanensis* (DSM 42132^T).

	ICN19	**ICN21**	***S. wuyuanensis* DSM 42132**
ISP2			
Growth	good	sparse	good
colony colour	beige red	sand yellow	mouse grey
aerial mycelium	traffic white	none	light grey
soluble pigment	none	none	none
ISP3			
Growth	good	good	good
colony colour	light pink/ antique pink	azure blue	dusty grey
aerial mycelium	cream	light grey	silk grey
soluble pigment	none	none	none
ISP4			
Growth	good	sparse	good
colony colour	ivory	ivory	squirrel grey
aerial mycelium	pure white	none	light grey
soluble pigment	none	none	none
ISP5			
Growth	good	sparse	good
colony colour	ivory	ivory	traffic grey
aerial mycelium	signal white	none	grey white
soluble pigment	none	none	none

ISP6			
Growth	good	sparse	good
colony colour	beige grey	sand yellow	brown beige
aerial mycelium	none	none	none
soluble pigment	terra brown	none	none
ISP7			
growth	sparse	sparse	good
colony colour	grey beige	ivory	mouse grey
aerial mycelium	none	none	light grey
soluble pigment	none	none	none

The three examined strains, ICN19, ICN21 and *S. wuyuanensis* (DSM 42132^T), differed in their physiological characteristics.

Table 26: Physiological properties of strains ICN19, ICN21 and the closest related type strain *S. wuyuanensis* (DSM 42132^T).

	ICN19	**ICN21**	***S. wuyuanensis* DSM 42132**
Tests			
ApiZym:			
Phosphatases alcaline	+	+	+
Esterase (C4)	+	+	+
Esterase lipase (C8)	+	+	+
Lipase (C14)	+/-	+	-
Leucine arylamidase	+	+	+
Valine arylamidase	+	+	+
Cystine arylamidase	+	+	+
Trypsin	+/-	+	+
Chymotrypsin	+/-	+	+
Phosphatase acid	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	+
α galactosidase	-	-	+/-
β galactosidase	-	-	-
β glucuronidase	-	-	-
α glucosidase	+	+/-	+
β glucosidase	-	-	+/-
N-acetyl-β-glucoseamidase	-	-	+
α mannosidase	+/-	+/-	+
α fucosidase	-	-	-

Carbon utilization			
Glucose	+	+	+
Arabinose	-	-	-
Sucrose	-	-	++
Xylose	(+)	++	++
Inositol	+	-	++
Mannose	-	-	-
Fructose	-	++	+
Rhamnose	-	-	-
Raffinose	-	-	-
Cellulose	-	-	-

While ICN19 and 21 showed a positive reaction on lipase and a negative on β-glucosidase and *N*-acetyl-β-glucoseamidase, *S. wuyuanensis* did not react on lipase but on the other two enzymes.

The main differences between the strains were observed in the ability of the utilization of different carbon sources. In contrast to *S. wuyuanensis*, ICN19 and 21 could both utilize sucrose. ICN19 and *S. wuyuanensis* were able to use inositol as carbon source and fructose was only utilized by ICN21 and *S. wuyuanensis* (Table 26).

3.3.2. Secondary metabolites produced by marine actinobacteria from India

ICN4, 16, 18, 19, 21, 26 and 28 were screened for bioactive secondary metabolites. Like all actinobacteria in this study, the strains were cultivated in the production media 5254 and 5294 prepared with and without artificial sea water. After cultivation and preparation of a raw extract, the bioactivity was tested using serial dilution assay. Interesting compounds were detected by fractionation experiments and identified by LS-MS analysis. The production rate of possibly new compounds was increased by media optimization, kinetics and "upscaling" experiments to finally isolate and purify the substance for the structure elucidation.

3.3.2.1. Results of bioactivity tests: serial dilution assays with standard and multi resistant test panel

After cultivation and harvesting of the secondary metabolites, the crude extracts of the different strain and media approaches were tested primarily on the standard test panel. The interesting extracts were afterwards also screened on a multi resistant test panel (Table 27).

Most of the extracts showed inhibitory effects against Gram-positive bacteria and fungal strains from the standard test panel. However, some of the strains also produced compounds showing bioactivity

against Gram-negative bacteria (appendix). The interesting extracts were additionally screened in the multi resistant test panel.

Table 27: MIC of the extracts from the isolated actinobacteria from marine and rhizosphere sediment from mangroves in India against the multi resistant test panel in different media: [4] 5254 medium; [5] 5254 sea water medium; [6] 5294 medium; [7] 5294 sea water medium.

	Fungi	**Gram +**				**Gram -**				
	C. albicans (DSM 1665)	*S. aureus* Newman*	*S. aureus* N315 (DSM 11822)	*E. faecium* (DSM 20477)	*E. faecium* (DSM 17050)	*P. aeruginosa* PA14 (DSM 19882)	*E.coli* (DSM 1116)	*E.coli* WT-3	*E.coli* XL-1 blue	*E.coli* ESBL (DSM 22664)
ICN16[5]	-	F	E	G	G	-	-	-	A	-
ICN18[4]	-	F	F	E	C	-	-	-	A	-
ICN18[6]	C	H	H	H	H	D	F	G	G	-
ICN19[5]	G	E	B	C	A	-	-	-	-	-
ICN19[6]	H	H	H	-	-	-	B	B	C	-
ICN21[6]	H	-	-	-	-	-	-	-	B	-
ICN26[4]	-	D	D	C	A	-	-	-	-	-
ICN28[4]	-	D*	D	E	E	-	-	-	-	-
ICN28[5]	F	B*	A	C	-	-	-	-	-	-

Most of the extracts showed a high number of inhibitions in the standard as well as in the multi resistant test panel. Therefore, a few were chosen for further fractionation experiments.

3.3.2.2. Interesting compounds produced by marine actinobacteria from India

Additionately to the fact that most of the determined bioactivities could be correlated to known compounds or not be reproduced in the fractionation experiment, in some cases, the amount of produced bioactive compound was too low or the ionization was very poor to work further on the isolation of these substances. Only strain ICN21 was chosen for broader metabolite analysis.

3.3.2.2.1. Strain ICN21: producer of staurosporine

The biological activity against *Candida albicans* was assigned to a compound with a mass of 467.2079 $[M+H]^+$m/z, a retention time of 7.5 min and a significant UV absorption (Figure 45).

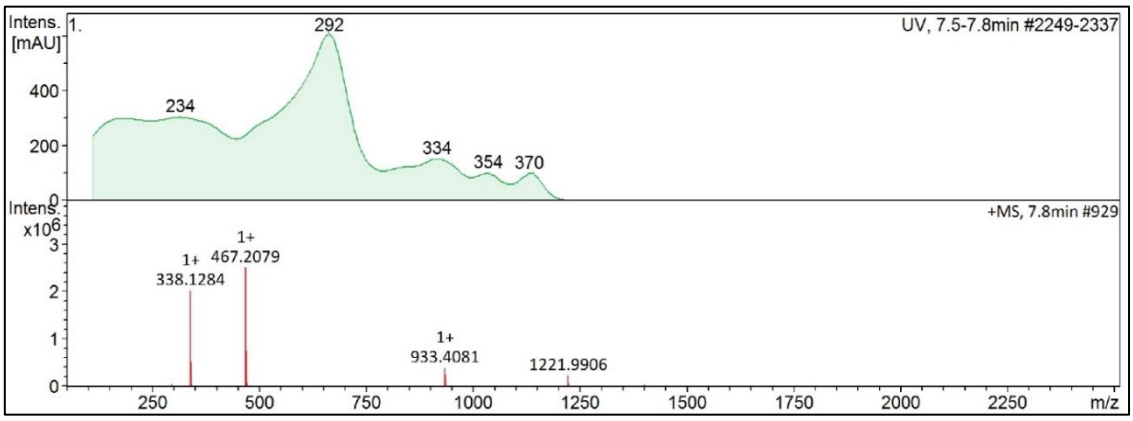
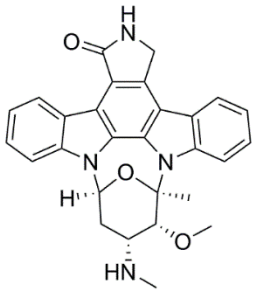


Figure 45: LC-MS run of the crude extract of ICN21 in 5254-SW medium. UV absorption and mass spectra of the bioactive compound with a mass of 467.2079 $[M+H]^+$ *m/z*.

Using the Dictionary of Natural Products (DNP) the compound was identified to be staurosporine ($C_{28}H_{26}N_4O_3$) (Figure 46). Staurosporin was described amongst other activities to have inhibitory effects against fungi and yeasts (Omura et al. 1977). Additionately, staurosporine was found to be produced by the taxonomically closely related strain ICN19.



Staurosporine

Figure 46: Chemical structure of staurosporine.

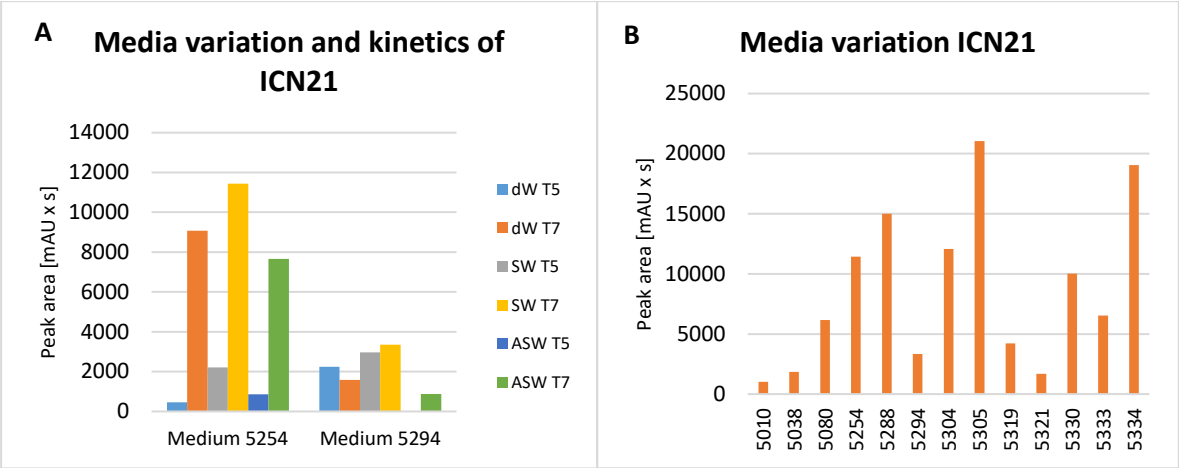


Figure 47: Media variations and kinetics of ICN21. To detect the production of staurosporin (masses 467.2079 $[M+H]^+$*m/z*, the peak area was determined. A: The media variation and kinetics of the strain ICN21 was done in the screening media for actinobacteria 5254 and 5294 with dest. water (dW), sampled sea water of the coast of Neuharlingersiel (SW) and artificial sea water (ASW) and was harvested at day 5 (T5) and 7 (T7) after inoculation. B: For amplified media variation of the strain ICN21 was cultured in the media: 5010, 5038, 5080, 5254, 5288, 5294, 5304, 5305, 5319, 5321, 5330, 5333 and 5334.

Figure 47 A and B show the media variations and kinetics for the best production of staurosporine. For most of the water combinations medium 5254 at day 7 showed the highest production of the compound. However, medium 5254 with addition of sampled sea water proved to be the most efficient combination. Further media variations with broader media used as common media for the production of secondary metabolites showed the medium 5305 to be the best choice for effective production of staurosporine by the strain ICN21.

3.4. Part 4: Actinobacteria from deep sea samples from the North Atlantic Ocean: Isolation, taxonomic characterization and screening for new bioactive compounds

The deep sea is one of the most unexplored places in the wold. On an expedition with the research vessel “Sonne”, some deep sea sediment samples and sponges were collected from 1092 m depth. It was possible to isolate 4 actinobacterial strains from these samples. These strains were phylogenetically characterized and screened for the production of biologically active secondary metabolites.

3.4.1. Characterization

Strains A-, B- and C-Sed $H10^{-3}$ and ASO4 wet were isolated from deep sea sediments from the North Atlantic Ocean. To classify these strains within their phylogeny, 16S rRNA as well as MALDI-TOF and RiboPrinter® analyses were done to find the closest relatives of these strains.

3.4.1.1. Characterization of strains A-, B- and C-Sed H10^{-3}: Phylogeny, morphology and physiology

Using 16S rRNA sequencing, the strains A-, B- and C-Sed H10^{-3} were classified as *Streptomyces* strains. This phylogenetic group consists of a plethora of strains which are closely related. The NCBI "Blast" search showed a 99 % identity of the 16S rRNA sequence with 35 other *Streptomyces* type strains (appendix). All of these strains are shown in the phylogenetic tree in figure 48. All three strains are very closely related to each other (between 99 and 100% homology in the 16S rRNA sequence), with strains A- and C-Sed H10^{-3} being the closest relatives. However, because of the high number of related type strains it was decided to stop working on the taxonomic description of the *Streptomyces* strains A-, B- and C Sed H10^{-3}.

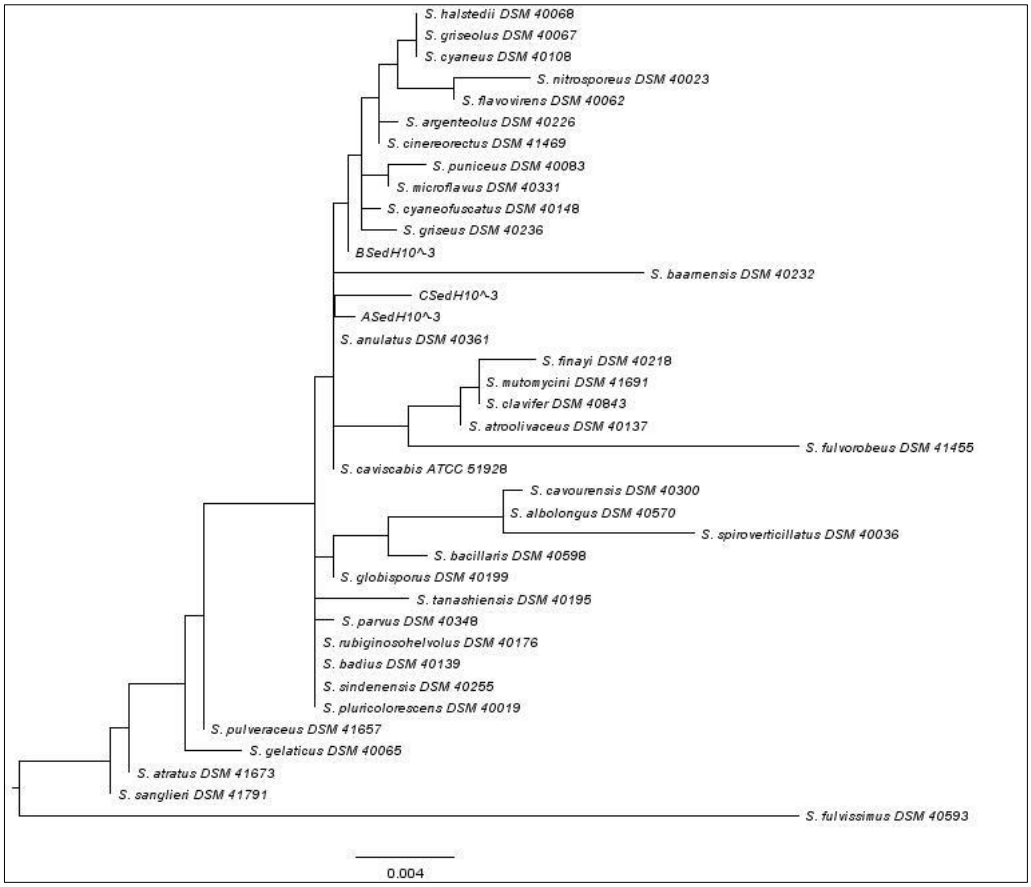


Figure 48: Phylogenetic tree based on the 16S rRNA sequence of strains A-, B- and C-Sed H10^{-3} and their closest related type strains. All type strains have more than 99% homology in the 16S rRNA sequence to the Sed H10^{-3} strains and were detected via "Blast" tool on NCBI and the tree was calculated using GGDC.

Because of the close relationship between strains A-, B- and C-Sed H10^{-3} in the 16S rRNA sequence, morphological and physiological parameters were examined, to distinguish them from each other.

Table 28: Comparison of the morphology (growth, colony colour, aerial mycelium and soluble pigments on ISP plates) of the isolated strains: A-, B- and C-Sed H10^{-3}.

	A-Sed H10^{-3}	B-Sed H10^{-3}	C-Sed H10^{-3}
ISP2			
Growth	good	good	good
Colony colour	colourless	brown beige	honey yellow, ochre yellow
Aerial mycelium	sparse	silver grey, grey white	sparse
Soluble pigment	none	none	none
ISP3			
Growth	good	good	good
Colony colour	n.d.	green beige	honey yellow, ochre yellow
Aerial mycelium	stone grey, pure white	mouse grey, platinum grey	agate grey, dusty grey
Soluble pigment	ochre yellow	sand yellow	ivory
ISP4			
Growth	good	sparse	good
Colony colour	ivory, lemon yellow	brown beige, clay brown	ochre yellow, curry
Aerial mycelium	sparse	none	dusty grey, light grey
Soluble pigment	none	none	none
ISP5			
Growth	good	good	good
Colony colour	sulfur yellow	lemon yellow	sulfur yellow
Aerial mycelium	none	sparse	sparse
Soluble pigment	none	none	none
ISP6			
Growth	sparse	none	good
Colony colour	n.d.	none	beige
Aerial mycelium	none	none	none
Soluble pigment	none	none	none
ISP7			
Growth	sparse	good	good
Colony colour	colourless	curry, khaki grey	curry, khaki grey
Aerial mycelium	none	oyster white	oyster white
Soluble pigment	none	lemon yellow	sand yellow

All three strains showed very similar growth and colony colours on the different ISP media. The colony colour differed in diverse shades of yellow and beige as well as grey areal mycelia, if existent. However, the growth and production of areal mycelia differentiated between the strains. Strain A-Sed H10^{-3} only formed areal mycelium on ISP3, but only sparse growth on ISP6 and 7, whereas B-Sed H10^{-3} produced areal mycelium on ISP2, 3 and 7 and did not grow on ISP6. Strain C-Sed H10^{-3} formed areal mycelium on media ISP3, 4, and 7 and grew well on all ISP media. A yellow to ivory soluble pigment was detected on ISP3 medium for every strain and on ISP7 medium for strains B- and C-Sed H10^{-3} (Table 28).

Table 29: Physiological properties of strains A-, B- and C-Sed H10^{-3}.

	A-Sed H10^{-3}	B-Sed H10^{-3}	C-Sed H10^{-3}
Tests			
ApiZym:			
Phosphatases alcaline	+	+	+
Esterase (C4)	+	+	+
Esterase lipase (C8)	+	+	+
Lipase (C14)	+/-	+	+
Leucine arylamidase	+	+	+
Valine arylamidase	+	+	+
Cystine arylamidase	+/-	+/-	-
Trypsin	-	+/-	-
Chymotrypsin	-	-	-
Phosphatase acid	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	+
α galactosidase	+/-	-	-
β galactosidase	+	+/-	+
β glucuronidase	-	-	-
α glucosidase	+	+/-	+
β glucosidase	+	+/-	+
N-acetyl-β-glucoseamidase	+	+	+
α mannosidase	+	+	+
α fucosidase	-	-	-
Carbon utilization			
Glucose	+	+	+
Arabinose	+	+	+
Sucrose	++	-	-
Xylose	++	++	++
Inositol	-	(+)	-
Mannose	++	++	+
Fructose	++	+	+
Rhamnose	++	+	+
Raffinose	-	-	-
Cellulose	-	-	-

Using ApiZym® tests the enzymatic activity of the strains were observed. All three strains showed the same enzymatic pattern. A negative result was detected for chymotrypsin, β-glucuronidase as well as α-fucosidase, whereas a weak negative result was visible for cystine arylamidase, trypsin and α-galactosidase. All of the other enzymes tested showed a weak positive result for strains A-, B- and C-Sed H10^{-3} (Table 29).

Also the possibility of carbon utilization of the three isolated deep sea strains was very comparably. All strains were able to utilize glucose, arabinose, xylose, mannose, fructose and rhamnose. Raffinose

and cellulose was not exploited by any strain. However, strain A-Sed $H10^{-3}$ was able to use sucrose as carbon source whereas the other two strains were not able to use this substrate (Table 29).

3.4.1.2. Characterization of strain ASO4 wet: Phylogeny, morphology and physiology

In contrast to the other three isolates from the deep sea samples, strain ASO4 wet showed a high potential to be a new species. Its closest relatives are the strains *S. karpasiensis* (98, 94%), *S. glycovorans* (98%) and *S. abyssalis* (98%) with less than 99% identity in the 16S rRNA sequence (Table 30). So these strains were ordered from the DSMZ for further analyses for the strain characterization as well as MALDI-TOF, RiboPrinter® and DNA-DNA hybridization (DDH) analyses.

Table 30: Closest relatives of the *Streptomyces* strain ASO4 wet after using the "Blast" tool on NCBI. . Based on the homology of the 16S rRNA sequence.

ASO4 wet	
S. karpasiensis (98,94%)	DSM 42068^T
S. glycovorans (98%)	DSM 42021^T
S. abyssalis (98%)	DSM 42024^T

With the help of the MALDI-TOF analysis (Figure 49) the very close relationship between the new isolated strain ASO4 wet and the type strain *S. karpasiensis* was evident (the same separation was detected in the phylogenetic tree based on the 16S rRNA sequence). To verify that the new strain ASO4 wet constituted as a new species, the strains were analyzed using RiboPrinter® analysis.

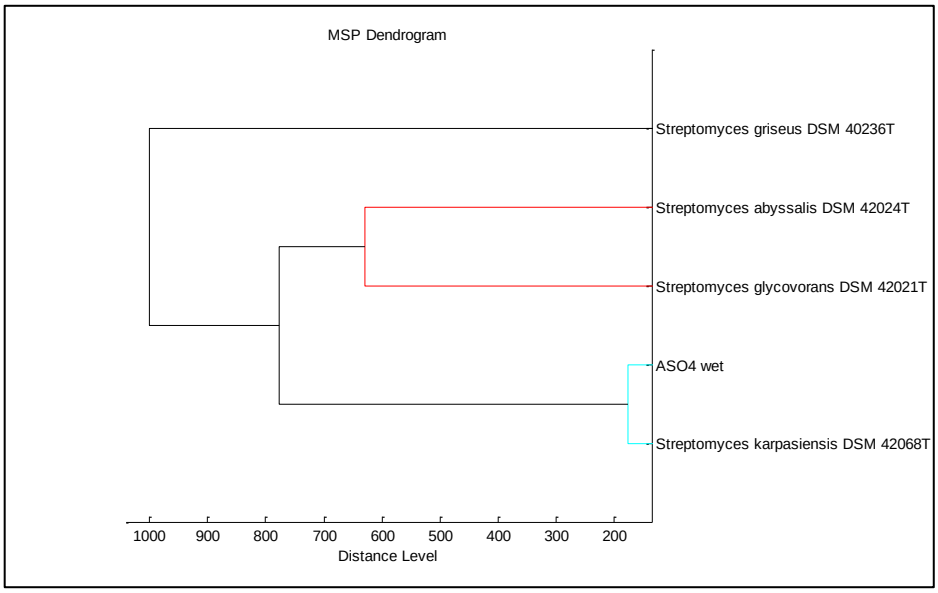


Figure 49: MALDI-TOF dendrogram of the *Streptomyces* strain ASO4 wet and its closest related type strains: *S. abyssalis* (DSM 42024^T), *S. glycovorans* (DSM 42021^T) and *S. karpasiensis* (DSM 42068^T). *S. griseus* (DSM 40236^T) was used as outgroup.

The comparison of the fingerprints using the RiboPrinter® software visualized the differences between the strains (Figure 50). All four strains showed a diverse band pattern. Especially the differences between the type strain *S. karpasiensis* and the new isolated ASO4 wet indicated the latter to be a new species.

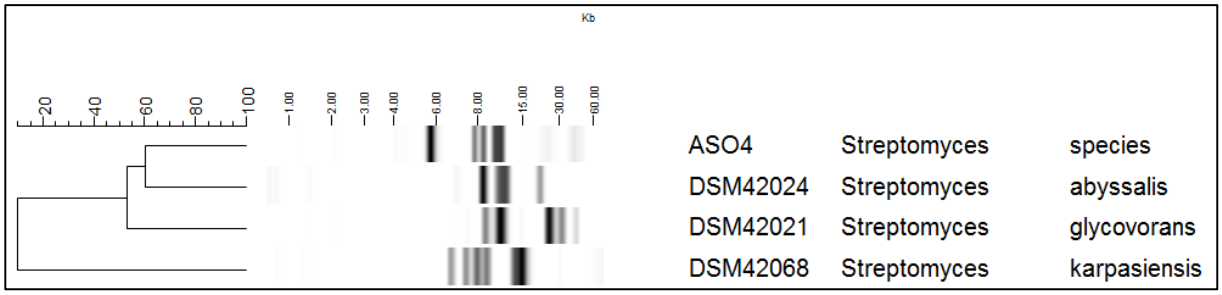


Figure 50: RiboPrinter® analysis of the *Streptomyces* strain ASO4 wet and its closest related type strains: *S. abyssalis* (DSM 42024^T), *S. glycovorans* (DSM 42021^T) and *S. karpasiensis* (DSM 42068^T).

The DNA-DNA hybridization analysis of strain ASO4 wet and its closest relatives yielded a value of 35.8/28.2 % against *S. abyssalis*, 40.5/44.4% against *S. glycovorans* and 40.4/54.7% against *S. karpasiensis* (Table 31). Therefore ASO4 wet is a new species, the closest relative of which is *S. kapasienesis*. Furthermore, a GC value of 72.4% was detected

Table 31: DNA-DNA hybridization (DDH) analysis of the *Streptomyces* strain ASO4 wet and its closest related type strains: *S. abyssalis* (DSM 42024^T), *S. glycovorans* (DSM 42021^T) and *S. karpasiensis* (DSM 42068^T). All hybridizations were performed twice.

DDH value against strain ASO4 wet (%)	
S. karpasiensis (DSM 42068^T)	40.4 / 54.7
S. glycovarans (DSM 42021^T)	40.5 / 44.4
S. abyssalis (DSM 42024^T)	35.8 / 28.2

To separate strains from each other, additionally to the genome, the morphology and physiology like the carbon utilization or the enzymatic activities were a significant marker.

Table 32: Comparison of the morphology (growth, colony colour, aerial mycelium and soluble pigments on ISP plates) of strains ASO4 wet *S. karpasiensis* (DSM 42068^T), *S. glycovorans* (DSM 42021^T) and *S. abyssalis* (DSM 42024^T).

	ASO4 wet	*S. karpasiensis*	*S. glycovorans*	*S. abyssalis*
ISP2				
Growth	good	good	sparse	good
Colony colour	light ivory	beige	n.d.	papyrus white/ ruby red
Aerial mycelium	none	none	none	papyrus white
Soluble pigment	none	none	none	none
ISP3				
Growth	good	good	good	good
Colony colour	light ivory	oyster white	papyrus white	grey white
Aerial mycelium	grey white	none	none	grey white
Soluble pigment	none	none	none	none
ISP4				
Growth	good	good	good	good
Colony colour	ivory	ivory	papyrus white	brown beige
Aerial mycelium	none	none	none	brown beige
Soluble pigment	none	none	none	none
ISP5				
Growth	good	good	good	good
Colony colour	light ivory	oyster white	papyrus white	papyrus white
Aerial mycelium	none	none	none	brown beige
Soluble pigment	none	none	none	none
ISP6				
Growth	sparse	sparse	good	good
Colony colour	sandy yellow	beige	papyrus white	papyrus white
Aerial mycelium	none	none	none	none
Soluble pigment	none	none	none	none
ISP7				
Growth	sparse	good	good	good
Colony colour	nut brown	beige	dive brown	clay brown/ green brown
Aerial mycelium	light grey	none	none	clay brown
Soluble pigment	none	none	none	none

The morphological data of the isolated deep sea strain ASO4 wet revealed ASO4 wet to be closely related to the three strains *S. karpasiensis*, *S. glycovorans* and *S. abyssalis*. The highest conformity was detected with its closest relative, *S. karpasiensis*. Both strains showed good growth on media ISP2, 3, 4 and 5 while *S. glycovorans* did not grow on ISP 2 medium. *S. abyssalis* grew well on every medium. However, ASO4 wet and *S. karpasiensis* did only grow sparsely on ISP6 medium while the other two strains grew well. The colony colour differed between white and beige up to yellow nuances on ISP2, 3, 4, 5 and 6 media and a brown colour on ISP7. The main difference between the deep sea isolate and the *S. karpasiensis* type strain was the production of areal mycelia. While ASO4 wet produced a grey white mycelium on ISP3 and 7, no mycelia production at all was detected for *S.*

karpasiensis. Furthermore, ASO4 wet showed just a sparse growth on ISP7 medium while its closest relatives grew well. Soluble pigments were not detected in any of the approaches (Table 32).

Figure 51: Morphological comparison between the strains ASO4 wet, *S. karpasiensis* (DSM 42068^T), *S. glycovorans* (DSM 42021^T) and *S. abyssalis* (DSM 42024^T) on GYM plates.

On the direct comparison between the four strains (Figure 51), ASO4 wet, *S. karpasiensis* (DSM 42068^T), S. glycovorans (DSM 42068^T) as well as S. abyssalis (DSM 42024^T), the close relationship between these strains were evident. All strains had a white/beige colour and a rough colony surface. However, the strain ASO4 wet was the only strain producing dark brownish pigment on GYM medium.

Table 33: Physiological properties of the strains ASO4 wet, *S. karpasiensis* (DSM 42068^T), *S. glycovorans* (DSM 42021^T) and *S. abyssalis* (DSM 42024^T).

	ASO4 wet	*S. karpasiensis*	*S. glycovorans*	*S. abyssalis*
Tests				
ApiZym:				
Phosphatases alcaline	+	+	+	+
Esterase (C4)	+	+/-	+/-	+
Esterase lipase (C8)	+	+/-	+	+
Lipase (C14)	-	-	+	-
Leucine arylamidase	+	+	+	+
Valine arylamidase	+	+	+	+
Cystine arylamidase	+	+	+	+
Trypsin	+	+	+	-
Chymotrypsin	+	+	+	-
Phosphatase acid	+	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	+	+
α galactosidase	-	-	-	-
β galactosidase	+	-	+	+
β glucuronidase	-	-	+/-	+/-
α glucosidase	+	+	+	+
β glucosidase	-	+/-	+	+
N-acetyl-β-glucoseamidase	+	+	+	+
α mannosidase	+	+	+	+
α fucosidase	-	-	-	-
Carbon utilization				
Glucose	+	+	+	+
Arabinose	+	-	++	+
Sucrose	+	+	++	++
Xylose	++	++	++	++
Inositol	++	+	++	++
Mannose	(+)	+	-	+
Fructose	(+)	+	-	+
Rhamnose	+	+	-	+
Raffinose	++	+	++	++
Cellulose	-	+	-	-

The results of the enzymatic activity test via ApiZym helped to distinguish between the new isolate and its closest relatives, especially the relation to the type strain *S. karpasiensis* (DSM 42068^T). While ASO4 wet*, S. glycovorans* and *S. abyssalis* showed a positive reaction on β-galactosidase, *S. karpasiensis* did not show any activity. However, the deep sea isolate and *S. karpasiensis* did not react in the β-glucuronidase test, the other two strains showed an ambiguous reaction. Furthermore, ASO4 wet did not react in the β-glucosidase assay while *S. glycovorans* and *S. abyssalis* reacted positive and *S. karpasiensis* ambiguous (Table 33).

Concerning the use of carbon source the difference between the isolate ASO4 wet and *S. karpasiensis* became more distinct. ASO4 wet could use glucose, arabinose, sucrose, xylose, inositol, mannose, fructose and raffinose as carbon source, *S. karpasiensis* was not able to utilize arabinose. However, this strain was the only one which was able to use cellulose as carbon source (Table 33).

3.4.2. Secondary metabolites produced by marine actinobacteria from the deep sea

The four isolated strains, A-, B-, and C-Sed $H10^{-3}$ as well as ASO4 wet, were screened using the two standard media, 5254 and 5294 medium. However, because of the marine origin of the isolates, the strains were additionally screened on the same media charged with artificial sea water. After cultivation and isolation of the secondary metabolites, the bioactivity was tested via serial dilution assay on the multi resistant test panel.

3.4.2.1. Results of bioactivity tests: serial dilution assay with the multi resistant test panel

Table 34: MIC of the extracts from the isolated actinobacteria from sponge and sediment samples from the deep sea against the multi resistant test panel in different media: [4] 5254 medium; [5] 5254 sea water medium; [6] 5294 medium; [7] 5294 sea water medium.

	Fungi	**Gram +**				**Gram -**				
	C. albicans (DSM 1665)	*S. aureus* Newman*	*S. aureus* N315 (DSM 11822)	*E. faecium* (DSM 20477)	*E. faecium* (DSM 17050)	*P. aeruginosa* PA14 (DSM 19882)	*E. faecium* (DSM 1116)	*E.coli* WT-3	*E.coli* XL-1 blue	*E.coli* ESBL (DSM 22664)
A SedH 10^{-3} [4]	-	-	A	-	-	-	-	-	-	-
A SedH 10^{-3} [5]	-	-	-	-	-	-	-	-	-	-
A SedH 10^{-3} [6]	-	-	A	-	-	-	-	-	-	-
A SedH 10^{-3} [7]	-	-	-	-	-	-	-	-	-	-
B SedH 10^{-3} [4]	-	-	-	-	-	-	-	-	A	-
B SedH 10^{-3} [5]	-	-	A	-	-	-	-	-	A	-
B SedH 10^{-3} [6]	-	-	A	-	-	-	-	-	-	-
B SedH 10^{-3} [7]	-	-	A	-	-	-	-	-	-	-
C SedH 10^{-3} [4]	-	A	A	-	-	-	-	-	-	-
C SedH 10^{-3} [5]	-	B	A	-	-	-	-	-	-	-
C SedH 10^{-3} [6]	-	A	A	-	-	-	-	-	A	-
C SedH 10^{-3} [7]	-	B	A	-	-	-	-	-	-	-
A SO4 wet [4]	-	A	B	-	-	-	-	-	-	-
A SO4 wet [5]	-	-	-	-	-	-	-	-	-	-
A SO4 wet [6]	-	B	B	-	-	-	-	-	-	-
A SO4 wet [7]	-	-	-	-	-	-	-	-	-	-

After the cultivation of the strains in the different screening media, crude extracts were produced. Each crude extract was screened directly on a multi resistant test panel. Unfortunately, neither the strains A-, B- or C-Sed $H10^{-3}$ nor strain ASO4 wet produced any secondary metabolites with significant bioactivity (Table 34).

3.5. Part 5: Polyphasic description of *Streptomyces* strains JS360 and *S. davawensis*

Streptomyces davawensis and *S. cinnabarinus* were described to be very close relatives and both producer of the riboflavin (vitamin B2) analogue roseoflavin. Because of this it was not sure whether they really do belong to the same species. Additionally, *Streptomyces* strain JS360 (*S. cinnabaragriseus*) is the producer strain of the biologically active cinnabaramides and was misleadingly described as *S. cinnabarinus* strain. To describe *S. cinnabaragriseus* as a new and separate species and for the validation of *S. davawensis* being a separate species, all strains were analyzed morphologically, physiologically and phylogenetically.

3.5.1. Phylogenetic analysis

To detect the phylogenetic relationship between the *Streptomyces* strains JS360 (*S. cinnabaragriseus*), *S. davawensis* and *S. cinnabarinus*, 16S rRNA as well as MALDI-TOF analyses were done. The 16S rRNA analyses of the three strains were done by Prof. Peter Kämpfer and his team of the University of Gießen. They detected the closest relatives with more than 98.5% similarity in the 16S rRNA sequence (appendix). These 23 *Streptomyces* type strains were afterwards used for construction of a phylogenetic tree based on the MALDI-TOF fingerprints as well as for the comparison of the morphological und physiological characteristics.

Due to the phylogenetic tree based on the MALDI-TOF finger prints it is clearly evident that strains *S. davawensis* (JCM 4913 / DSM 40467^T) and *S. cinnabarinus* (DSM 40467^T) are closely related and clustered together with 11 further *Streptomyces* strains in a huge group (green). In contrast, *Streptomyces* strain JS360 is the closest relative of *S. griseoruber* (DSM 40281^T) and together with *S. corchorusii* (DSM 40340^T) the three strains build a separate cluster (blue) (Figure 52).

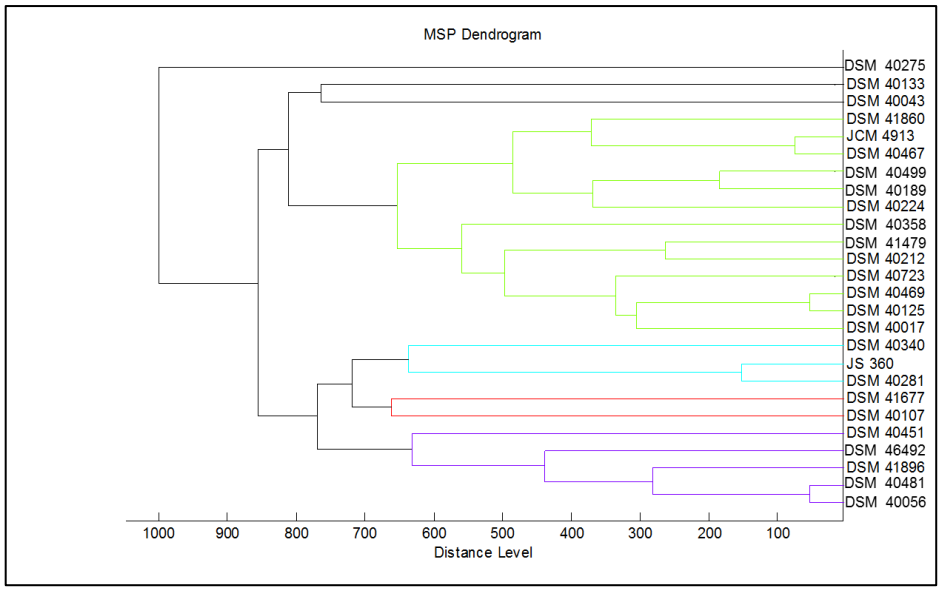


Figure 52: MALDI-TOF dendrogram of the *Streptomyces* strains JS360 (*S. cinnabaragriseus*), *S. davawensis* (JCM 4913 / DSM 101723), *S. cinnabarinus* (DSM 40467^T) and their closest relatives. All reference strains were detected via 16S rRNA analysis.

3.5.2. Morphological and cultural observations

White, beige grey or cream aerial mycelium were observed on most of the IPS media for the strains *S. cinnabarinus, S. davawensis* and *S. cinnabaragriseus* (JS360). While ISP6 was the only medium where none of the strains produced areal mycelium, *S. davawensis* also produced no areal mycelium on ISP 2 and strain JS360 additionally showed sparse areal mycelium growth on ISP5 and 7. The spores of *S. davawensis* and *S. cinnabarinus* form fragmented, almost parallel laying spore chains of slightly rough spores. In contrast, the surface of the spores of JS360 showed an irregular fragmentation which seemed to be desiccated and the spore chains appeared disordered (Figure 53). The colour of the substrate mycelium of *S. davawensis* differed between sad yellow (ISP2), light pink (ISP3), red orange (ISP4), black red (ISP5 and 7) and green brown (ISP6). The colours of JS360 (*S. cinnabaragriseus*) were detected to be between honey yellow (ISP2), vermillion (ISP3), red orange (ISP4), black red (ISP5, 7) and beige grey (ISP6). However, some differences between the strains *S. davawensis* and *S. cinnabaragriseus* (JS360) and the strain *S. cinnabarinus* were observed. The colour of the strain *S. cinnabarinus* on medium ISP2 was brown red whereas the other two strains had a yellow colour and on ISP5 *S. cinnabarinus* showed a very light red colour instead of the red black colour of the compared strains. Another significant difference was detected in the production of soluble pigments. A reddish pigment was observed in the media ISP2 and 4 produced by *S. cinnabarinus*, on ISP3 from *S. cinnabarinus* and *S. davawensis*, an olive brown pigment from all three strains on ISP6 and also on ISP7 produced by *S. davawensis* and *S. cinnabaragriseus* (JS360) (Table 35).

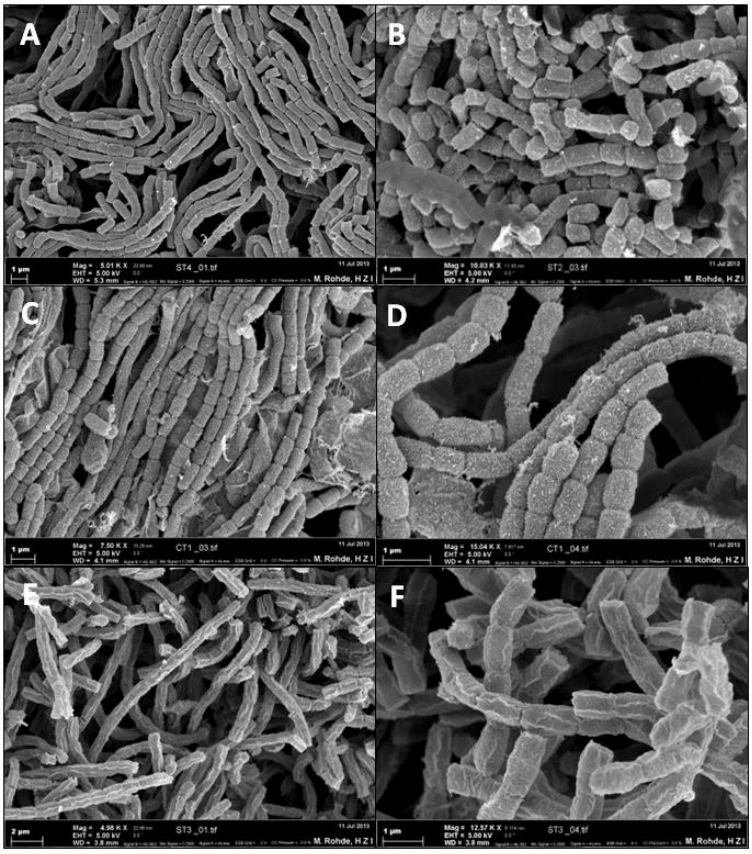


Figure 53: Scanning electron microscopy displaying the morphology of the *Streptomyces* strains *S. cinnabarinus*, *S. davawensis* and JS360. A, B*: S. cinnabarinus* (DSM 40467^T), C, D*: S. davawensis* (DSM 101723), E, F: JS360 (*S. cinnabaragriseus*). Cultivation was carried out on medium ISP3 at 28 °C for 14 days. (Bar = A, B, C, D, F: 1 µm; E: 2 µm). (Taken by Prof. Dr. Rohde, HZI Braunschweig, Germany)

Table 35: Morphology (ISP + carbohydrates) and physiology of *S. cinnabarinus* (DSM 40467^T), *S. davawensis* (DSM 101723) and JS360 (*S. cinnabaragriseus*).

	S. cinnabarinus	***S. davawensis***	**JS360 (*S. cinnabaragriseus*)**
ISP2			
Growth	good	good	good
Colony colour	brown red	sand yellow	honey yellow
Aerial mycelium	cream	none	beige grey
Soluble pigment	salmon pink	none	none
ISP3			
Growth	good	good	good
Colony colour	salmon pink	light pink	vermillon
Aerial mycelium	cream	signal white	beige grey
Soluble pigment	salmon pink	beige red	none
ISP4			
Growth	good	good	good
Colony colour	salmon pink	red orange	red orange
Aerial mycelium	cream	beige grey	beige grey
Soluble pigment	salmon pink	none	none

ISP5			
Growth	good	good	good
Colony colour	salmon pink	black red	black red
Aerial mycelium	sparse	beige grey	beige grey
Soluble pigment	none	none	none
ISP6			
Growth	good	decreased	decreased
Colony colour	olive drab	green brown	beige grey
Aerial mycelium	none	none	none
Soluble pigment	olive drab	olive brown	ocher brown
ISP7			
Growth	good	good	good
Colony colour	Salmon pink	black red	black red
Aerial mycelium	sparse	beige grey	beige grey
Soluble pigment	none	olive brown	olive brown

3.5.3. Physiology

The differences in the enzymatic activities were detected by using the ApiZym® test system (Table 36). All three strains showed a quiet similar enzymatic pattern. However, positive enzymatic reactions were detected against α-galactosidase only from *Streptomyces* strain JS360, against N-acetyl-β-glucoseamidase only from *S. davawensis* and α-fucosidase only from *S. cinnabarinus*

Glucose, arabinose, sucrose, inositol, mannose, fructose, rhamnose and raffinose were utilized by all of the three strains*, S. cinnabarinus* (DSM 40467^T), *S. davawensis* (DSM 101723^T) and *Streptomyces* strain JS360 (*S. cinnabaragriseus*). In contrast to the both new strains, strain *S. cinnabarinus* was able to utilize cellulose but showed a negative result in the utilization of xylose (Table 36).

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Table 36: Physiological properties of *S. davawensis*, *S. cinnabarinus*, *Streptomyces* strain JS360 and their closest relatives: 1 *S. davawensis* DSM 101723^T, 2 *S. cinnabarinus* DSM 40467^T, 3 *S. cinnabaragriseus* DSM 101724^T, 4 *S. avermitilis* DSM 46492^T, 5 *S. flavovariabilis* DSM 41479^T, 6 *S. novaecaesareae* DSM 40358^T, 7 *S. alboniger* DSM 40043 ^{T}, 8 *S. cellostaticus* DSM 40189^T, 9 *S. bobili* DSM 40056^T, 10 *S. galilaeus* DSM 40481^T, 11 *S. griseochromogenes* DSM 40499^T, 12 *S. pseudovenezuelae* DSM 40212^T, 13 *S. phaeoluteigriseus* DSM 41896^T, 14 *S. atriruber* DSM 41860^T, 15 *S. resistomycificus* DSM 40133^T, 16 *S. yokosukanensis* DSM 40224^T, 17 *S. olivochromogenes* DSM 40451^T, 18 *S. corchorusii* DSM 40340^T, 19 *S. longwoodensis* DSM 41677^T, 20 *S. curacoi* DSM 40107^T, 21 *S. antibioticus* DSM 40234^T, 22 *S. canus* DSM 40017^T, 23 *S. ciscaucasicus* DSM 40275^T, 24 *S. griseorubiginosus* DSM 40469^T, 25 *S. phaeopurpureus* DSM 40125^T and 26 *S. griseruber* DSM 40281^T; all data from this study.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Tests																										
ApiZym:																										
Phosphatases alcaline	+	+	+	+/-	+	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase (C4)	+	+	+	-	-	-	+	-	-	-	-	-	-	+/-	-	-	+	+	-	+	+	+	-	+	+	-
Esterase lipase (C8)	+	+	+	-	-	+/-	+	-	+/-	+/-	+	-	-	+	-	+/-	+	+	+	+	+	+	-	+	+	+
Lipase (C14)	+	+	+	-	-	-	-	-	-	-	-	+	-	+/-	-	-	-	-	-	+	+/-	+/-	-	+	-	+/-
Leucine arylamidase	+	+	+	+	+	+	+	+	+/-	+	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+
Valine arylamidase	+	+	+	+/-	+	+	+	+	-	+	+	+/-	-	+	+	+	-	+	+	+	+	+	-	+	+	+
Cystine arylamidase	+	+	+	+/-	+/-	+/-	+	-	+	-	+	-	-	+/-	-	+	+/-	+	+/-	+	+/-	+/-	-	+	+/-	+/-
Trypsin	+	+	(+)	+/-	-	-	+	-	-	+	+	-	-	+	+	+	-	+/-	-	+	+/-	-	-	+	-	-
Chymotrypsin	-	-	-	-	-	-	+/-	+	-	-	+	-	-	+	-	+	-	+/-	-	+	-	-	-	-	-	-
Phosphatase acid	+	+	+	+	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	+	-	+	-	+	+/-	-	+/-	+	-	-	-	-	+/-	+	+	+	+	+	+	+	+	+	+
α galactosidase	-	-	+	-	-	+/-	+	+	+/-	+	-	+/-	-	+/-	-	+/-	+	+	+/-	+	+	+	-	+	+	+/-
β galactosidase	+	+	+	+/-	+	+	+	-	+/-	+	+	+	+/-	+	+	+/-	+	+	+	+	+	+	+	+	+	+
β glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α glucosidase	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β glucosidase	+	+	+	-	+/-	+	+	+	+/-	+	+	+	+/-	+	+	+	+	+/-	+/-	+	+	+	+	+	+	+
N-acetyl-β-glucoseamidase	+	-	-	+	-	+	+	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+/-	-	+	+	-
α mannosidase	+	+	+	+	+/-	+	+	-	-	+	-	+	-	+	-	+/-	+	+	-	+	+	+	-	+	+	+
α fucosidase	-	+	-	-	-	-	-	-	-	-	-	+/-	-	+/-	-	+/-	+	+	-	-	-	+	+	-	-	-

Carbon utilization																										
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	(+)	+	-	+	+	+	++	+
Sucrose	+	+	+	-	+	+	-	(+)	+	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	+	-
Xylose	+	-	+	-	-	+	-	(+)	+	-	-	+	++	+	+	++	-	-	(+)	+	-	-	-	-	++	-
Inositol	+	+	+	(+)	+	+	+	+	+	+	+	+	++	+	++	++	+	+	-	+	+	-	+	+	++	-
Mannose	+	+	+	+	+	+	+	+	-	-	+	+	++	++	++	-	+	+	(+)	+	(+)	+	+	+	+	-
Fructose	+	+	++	+	+	+	-	+	+	+	+	+	++	++	++	+	+	+	-	+	(+)	+	+	+	++	(+)
Rhamnose	+	+	+	+	+	+	-	+	+	+	-	+	+	++	+	++	+	+	-	-	(+)	+	+	+	++	(+)
Raffinose	+	+	+	(+)	+	+	-	+	+	+	+	+	-	+	++	++	-	-	-	-	-	-	+	+	++	-
Cellulose	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-

4. Discussion

4.1. Marine myxobacteria

This part of the study was based on the publications of Jiang et al. (2010) and Brinkhoff et al. (2012) about the "Marine Myxobacteria Cluster" (MMC). Using molecular approaches, they found a specific rRNA sequence in diverse marine samples, which are on the one hand related to the 16S rRNA sequences of known terrestrial myxobacteria but on the other hand phylogenetically different at high levels and therefore indicate "a phylogeographical separation between myxobacteria in the ocean and on land" (Jiang et al. 2010). The specific MMC sequence was isolated in marine sediments almost all over the world (Brinkhoff et al. 2012). However, to date, no MMC sequence harbouring myxobacteria could be isolated. Except the three "marine myxobacterial" families *Haliangium* (Fodou et al. 2002), *Plesiocystis* (Iizuka et al. 2003a) and *Enhygromyxa* (Iizuka et al. 2003b), which need a certain NaCl concentration to thrive (halophilic) but clustered within the terrestrial myxobacteria of the genus *Nannocystis*, only halotolerant bacteria which belong to the genera *Myxococcus*, *Angiococcus*, *Corallococcus* and *Cystobacter* were isolated (e.g. Li et al. 2002, Brinkhoff et al. 2012). Just as Brinkhoff et al. (2012), who isolated the two *Myxococcus* strains MX1 and MX2, in this study only halotolerant *Myxococcus* strains (WS 1.1, 2.1, 2.3, 3.3, 3.4, 4.1, 5.1, 9.1, 15.1, 21.3, 28.1 and 28.3) were isolated. These 12 strains clustered within the terrestrial myxobacteria and did not harbour the MMC sequence. However, this sequence was verified in almost every sample from the Wadden Sea besides algae and dry sand samples from the coast of Neuharlingersiel.

Basing on Zhang et al. (2005) myxobacteria which were isolated from marine habitats could be separated into three groups: the nonhalotolerant, the halotolerant and the halophilic isolates. The nonhalotolerant strains are not able to grow with salt concentrations higher than 1% (Reichenbach & Dworkin 1992) and have been isolated because of washed in myxospores from terrestrial myxobacteria. Meanwhile halotolerant isolates can grow with and without salt water and tolerate a wide range of NaCl concentrations. These strains are phylogenetically very closely related to terrestrial myxobacterial strains (Li et al. 2002) and may have evolved from their terrestrial relatives by adapting to the conditions of the marine environment. In contrast, the halophilic isolates, such as members of the families *Haliangium* (Foudou et al. 2002), *Plesiocystis* (Iizuka et al. 2003a) and *Enhygromyxa* (Iizuka et al. 2003b) were classified as a new myxobacterial group because of phylogenetical distance as well as the morphologic differences. Using this description, the 12 *Myxococcus* isolates from the Wadden Sea belong to the group of halotolerant myxobacteria. Strains WS 3.4, and 4.1 formed orange to yellow fruiting bodies and swarms on media containing up to 2.5% NaCl and strain WS 5.1 even build fruiting bodies up to 10% NaCl. This wide range of sodium chloride

tolerance is very impressive especially compared with the optimum NaCl concentration of the described halophilic myxobacteria between 1.0- 3.0 % (Foudou et al. 2002; Iizuka et al. 2003a; Iizuka et al. 2003b). However, Zhang et al. (2005) also described a changing growth strategy of the halotolerant myxobacteria. With higher salt concentrations, on agar plates the cells stopped to form fruiting bodies and the swarming cells changed their colour to light tan, the cells itself shortened into spheres of the size of myxospores and in liquid media they showed cell independent growth as well as the formation of myxospores directly from vegetative cells. Almost the same scenario was detected during the NaCl tolerance test of the Wadden Sea isolates. All strains formed colourless fruiting bodies on media containing up to 10% sodium chloride without any swarming and the cells seemed weak. Furthermore, similar to the description of Zhang et al. (2005), the WS–strains clustered phylogenetically within their cultured terrestrial relatives. However, most of the strains did not have the exact same 16S rRNA sequence and build a separate cluster in between. This close relationship was also detected during the screening of the production of new bioactive secondary metabolites of the 12 isolated *Myxococcus* strains. All detected biological activities could be correlated to known substances produced by terrestrial myxobacteria in in-house database at HZI, "Myxobase".

In sum, this study reflects the findings of other working groups (e.g. Li et al. 2002, Brinkhoff et al. 2012) as it was only possible to isolate halotolerant or even halophilic (Foudou et al. 2002; Iizuka et al. 2003a; Iizuka et al. 2003b) myxobacteria from marine habitats. Unfortunately none of these myxobacteria harbour the MMC sequence. Phylogenetically, they clustered within their terrestrial relatives with a high distance to the bacterial MMC clones from the marine samples (Brinkhoff et al. 2012). To isolate "real" marine myxobacteria all scientists agree that on the one hand a sample is needed from the deep oceans and on the other hand new isolation techniques, which mimic the natural environment of the samples, have to be established. Furthermore, because of the unknown lifecycle and morphology of marine myxobacteria, isolation techniques should not focus on characteristic swarms and fruiting bodies but consider other unusual bacterial colonies.

4.2. Description of new (marine) actinobacteria

"Taxonomic information is essential, as it enables scientists to understand the biodiversity and relationships among living organisms from different ecosystems" (Ramasamy et al. 2014; Gevers et al. 2005). With the classification of bacteria by basic phenotypic markers like the description of morphology, growth requirement or pathogenic potential, Lehmann and Neumann started the age of microbial taxonomy in 1896. However, during the last century physiological and biochemical

properties (Orla-Jensen 1909; Buchanan 1955), chemotaxonomy (Minnikin et al 1975), numerical taxonomy (Brenner et al. 1969), DNA-DNA hybridization (Johnson 1991) and the comparison of the 16S rRNA sequence with described type strains (Ramasamy et al. 2014; Gürtler & Mayall 2011; Coenye & Vandamme 2004; Konstantinidis & Tiedje, 2007) were added to the common description approaches. In sum, to date the description of a new prokaryotic strain is a polyphasic combination of phenotypic, chemotaxonomic and genotypic analyses (Vandamme et al. 1996, Stackebrandt et al. 2002, Tindall et al. 2010).

In this study, the 16S rRNA sequence was used as a first marker for the search of novel marine actinobacteria. Basing on Stackebrandt et al. (2002) a new genus or a new species could be described if the isolate had 16S rRNA identity values of 95 and 97%, respectively, compared to their closest phylogenetic neighbour. In 2006 this value was increased to 98.7% (Stackebrandt & Ebers). However, such high values of 16S rRNA sequence identities like in the genus *Streptomyces and Micromonospora*, where different species have 16S rRNA similarities up to 99%, have to be correlated with DNA-DNA hybridizations (Rosselló-Móra 2006). Wayne et al. (1987) described isolates with less than 70% homology as distinct species. Moreover, in such genotechnological tight relationships, the morphological and physiological descriptions have to be focused.

Most of the Guam strains as well as all ICN strains and the three isolated deep sea strains A-, B- and C-Sed $H10^{-3}$ belong to the genera *Streptomyces* or *Micromonospora*. These strains had a 16Sr RNA sequence similarity of 99% to numerus type strains. Therefore, this study focused on the polyphasic taxonomic description of strains JS360, *S. cinnabarinus* and *S. davawensis* as well as the deep sea isolate ASO4 wet.

Micromonospora strain Guam 1582, the producer strain of the bioactive rakicidins A, B and E was further analyzed taxonomically using MALDI-TOF and compared to *Micromonospora chalcea* via RiboPrinter®. After 16S rRNA and MALDI-TOF analyses *M. chalcea* was detected to be the closest relative to strain Guam1582. Songsumanus et al. (2013) as well as Xie et al. (2011) described this strain as producer strain for rakicidin A and B. Using the RiboPrinter® analysis, which is able to compare different bacteria on strains level, strain Guam 1582 was identified as individual strain of the genus *Micromonospora*.

Furthermore, strains ICN19 and 21 were morphologically and physiologically compared to the type strain *Streptomyces wuyuanensis*. Strains ICN19 and 21 were 100% identical to each other and 99% to S. *wuyuanensis* due to the 16S rRNA sequence. However, during the morphological (growth on different agar plates) and physiological (ApiZym test) analyses, all strains showed different characteristics. One explanation for this observation was the sparse growth of ICN21 on the different

agar plates. Maybe the pre-culture was not in the same growth phase as the other two strains or the cells have even been not fit enough to grow well on the different plates. To be sure, the morphological analysis should be repeated and further analyses such as DNA-DNA hybridization as well as MALDI-TOF and RiboPrinter® analyses have to be done to describe ICN21 and ICN19 as new species.

4.2.1. Streptomyces strains *S. cinnabaragriseus* (JS360) and *S. davawensis*

In this study he phylogenetic relationship between the *Streptomyces* strains JS360, *S. davawensis* and the type strain *S. cinnabarinus* was analyzed.

Streptomyces davawensis was isolated in 1974 from a Philippine soil sample during a screening program for new antibiotics (Otani et al. 1974; Shinobu 1974). It was found to produce the red compound roseoflavin, which is named after its colour and the close structural relationship to the riboflavin (vitamin B_2) and biological active against Gram-positive bacteria. To date, roseoflavin is the only known natural riboflavin analogue which acts against bacteria Pedrolli et al. 2013. Vitamin analogues form a new interesting class of antibiotics. During the search for other producing strains, the 16S rRNA of *S. davawensis* was analyzed and *S. cinnabarinus* was found be a close phylogenetic relative and to be able to synthesize roseoflavin (Jankowitsch et al. 2011). After this success, other strains described as *S. cinnabarinus*, were screened for the production of this uncommon substance. As a result the metabolite profile of strain JS360, described as a *S. cinnabarinus* strain (Stadler et al. 2007), was analyzed. This strain was isolated from a soil sample collected in Japan and detected during a high through put screening program in a human 20S proteasome inhibitory assay (Stadler et al. 2004; Stadler et al. 2007). It was not able to produce roseoflavin, however, strain JS360 was described to be the producer strain of the cinnabaramides A-G (Stadler et al. 2007). These substances are proteasome inhibitors and can be used for the treatment of allergies, asthma as well as cancer (Kyle et al.2004). Furthermore, due their chemical structures, which are closely related to lactacystin and salinosporamide (a promissing compound isolated from the marine actinobacterium *Salinispora tropica*), it has a great potential to be used as anticancer drug (Stadler et al. 2004; Stadler et al. 2007).

To finally detect the relationship between strains *S. davawensis*, *S. cinnabarinus* and the *Streptomyces* strain JS360, the three strains were polyphasically analyzed during this thesis. The phylogenetic analyses and the tree constructed with MALDI-TOF fingerprints revealed the close relationship between the strains *S. davawensis* (JCM 4913 / DSM 40467^T) and *S. cinnabarinus* (DSM 40467^T). Additionately the phylogenetic distance to strain JS360 (*S. cinnabaragriseus*, DSM 101724^T)

which was misleadingly described as *S. cinnabarinus* (Stadler et al. 2007) was clearly evident. Strain JS360 was closely related to *S. griseoruber* (DSM 40281^T) and located in a different phylogenetic cluster. Furthermore, the shape of the spores and spore chains, which were quite similar only between *S. cinnabarinus* and *S. davawensis*, support the phylogenetic findings. During the morphological and physiological analyses not only the phylogenetic distance between strain JS360 (*S. cinnabaragriseus*) and both compared strains, but also between the closely related strains *S. cinnabarinus* and *S. davawensis*, became visible. Some significant differences between the colony colour and areal mycelium growth on ISP2, 5 and 7 as well as in the production of soluble pigments on ISP 2, 3, 4, 6 and 7 were observed. The physiological tests showed that in contrast to the other two strains, *S. cinnabarinus* was able to utilize cellulose but no xylose. However, during the enzymatic activity test, all three strains showed a similar enzymatic pattern besides the positive reaction against against α-galactosidase from *S. cinnabaragriseus* (JS360), against *N*-acetyl-β-glucoseamidase from *S. davawensis* and α-fucosidase from *S. cinnabarinus*.

In sum, based on the phylogenetic, morphological and physiological analyses conducted during this thesis, including the DNA-DNA hybridization results of the working group of Peter Kämpfer from the university of Gießen (data not shown), strain *S. davawensis* (DSM 101723^T) could be validly described as separate individual species and the strain JS360 as the new *Streptomyces* species *S. cinnabaragriseus* (DSM 101724^T).

4.2.2. *Streptomyces* strain ASO4 wet

During the course of this study three *Streptomyces* strains from deep sea sediment samples were isolated from 1092 m depth (A-, B- and C-Sed H10^{-3}) and one from a deep sea sponge (ASO4 wet) from the North Atlantic Ocean. Because of the close relationship between the strains of the genus *Streptomyces* (99% similarity within the 16S rRNA sequence between different species) this study was focused on strain ASO4 wet. With a 16S rRNA similarity of 98% to strains *S. glycovorans* (DSM 42021^T) and *S. abyssalis* (DSM 42024^T) as well as 98.94% to *S. karpasiensis* (DSM 42068^T), the strain seemed to be a promising new species.

Due to the morphological and physiological analyses ASO4 wet was revealed to be closely related to the type strain *S. karpasiensis*. *S. karpasiensis* was isolated from a soil sample collected from the Karpaz National Park in Magusa, northern Cyprus (Veyisoglu et al. 2014). In the direct comparison between the four strains on GYM medium the colonies of these two strains only differed in the production of melanin. Furthermore, except the mycelia production on all ISP media as well as a sparse growth on ISP7 medium of *S. karpasiensis*, both strains were detected to produce the same

colony colours. However, significant differences were noticed during the physiological tests, like the positive reaction of ASO4 wet on β-galactosidase and the ability to use arabinose as carbon source, while *S. karpasiensis* could utilize cellulose.

Using DNA-DNA hybridization with a value of 40.4 and 54.7 % against *S. karpasiensis*, respectively, and the comparison of the fingerprints, visualized via RiboPrinter®, it became clear that ASO4 wet could be described as a new distinct species of the genus *Streptomyces.*

During cultivation, strain ASO4 wet did not produce any interesting bioactive compounds. However, this strain was isolated from a marine sponge and after Blunt et al. (2011; 2012; 2013) most sponge associated bacteria are described to be produces of natural products. Therefore, maybe the cultivation conditions have to be adapted to the natural marine habitat of this strain, like a decrease of the cultivation temperature, cultivation approaches in the dark, co-cultivation with other bacteria isolated from this sponge or even an increase of the atmospheric pressure.

4.3. Bioactive substances isolated from marine actinobacteria

In general, it is very difficult to isolate new bioactive compounds from actinobacteria because over 50% of the antibiotics in clinical use today were produced from members of the genus Actinomycetales. Up to the year 2002, a sum of 20,000 antibiotics have been discovered of which 45% were produced by actinobacteria (Berdy 2005). Therefore, without a complete and updated database it is not possible to identify possibly new compounds as known ones. As a result much work was invested to detected bioactive compounds which were later on identified as rakicidins A, B and E, aloesaponarin II, 5-hxdroxyaloesaponarin II as well as staurosporine. However, in some cases the known compound could be correlated to a new activity or new microbial source.

4.3.1. Rakicidin A, B and E- known compounds, new bioactivity

In this study, rakicidins A, B and E were isolated from the marine *Micromonospora* strain Guam 1582. In general, *Micromonospora sp*. are known to be important producers of various antibiotic secondary metabolites such as gentamicin, kanamycin, microsporin or megalomycin (Genilloud et al. 2012) to name just a few. Songsumanus et al. (2013) as well as Xie et al. (2011) described *Micromonospora chalcea* as producer strain for rakicidin A and B and in other papers (e.g. McBrien et al. 1995) the producer strain was only described as *Micromonospora sp*.

Rakicidins A and B are well known substances which are described to inhibit tumour cell lines (Xie et al. 2011; Takeuchi et al. 2011; Yamazaki et al. 2007), whereas for rakicidin E no bioactivity has been described, yet (Oku et al 2014). With an IC_{50} value of 40 ng/mL and 200 ng/mL McBrien et al. (1995) described the cytotoxicity of rakicidins A and B against the murine cell line M109 (MADISON lung carcinoma 109), respectively. Because of its significant ability to induce hypoxia-selective cytotoxicity in solid cells and to induce cell death in TKI-resistant CML stem cells (Takeuchi et al. 2011; Yamazaki et al. 2007), rakicidin A is a highly interesting compound and other members of the rakicidin family are of ”particular interest for both, synthetic and medicinal studies” (Poulsen, 2011; Clement et al. 2015; Sang et al. 2014; Tsakos et al. 2016; Sang et al. 2016). Except rakicidin A, B and E, which were part of this study, the rakicidin family included two further compounds, rakicidin C and D. While rakicidin C did not show any bioactivity (Hu et al. 2000), rakicidin D was described to inhibit the invasion of murine carcinoma colon 26-L5 cells with an IC_{50} of 3.3 µg/mL (Igarashi et al. 2010). However, these two compounds were produced by *Streptomyces sp*. strains. Furthermore, the rakicidin family belongs to a rare structural group with the unusual amino acid, 4-amino-2, 4-pentadienoate which is only found to be produced by actinobacteria (Igarashi et al. 2010). To date, there are only two further classes of cyclic peptides reported to contain this unusual amino acid: the BE-43547 complexes (Nishioka et al. 1998) as well as the vinylamycin-microtermolide class (Igarashi et al. 1999; Carr et al. 2012; Oku et al. 2014). The compound vinylamicin is described as an antibiotic against Gram-positive bacteria including MRSA (Igarashi et al. 1999) whereas the microtermolides A and B show neither antibiotic nor antifungal activities (Carr et al. 2012). Contrary to the findings of Mc Brien et al. (1995) who stated that rakicidin A and B did not show any bioactive effect on bacteria or fungi, in this study it was shown that in liquid culture, also rakicidin A, B and E were selectively active against Gram–positive bacteria including the methicillin resistant *Staphylococcus aureus* N315 (MRSA) and the vancomycin resistant *Enterococcus faecium* DSM 17050 (VREF). Only a bioactivity against the Gram-positive bacterium *Kocuria rhizophila* was detected by Songsumanus et al. (2013) during the screening of a raw extract, before. These antibiotic activities, especially against the multi resistant MRSA and VREF strains show new aspects of the medical impact of the rakicidin family. However, because of a two to 16 times higher bioactivity value against the murine cell line than against the tested multi resistant bacteria, further studies will probably focus on the cytotoxicity instead of the antibiotic effects of rakicidin A, B and E. Especially the very interesting activities of rakicidin A against cancer cell lines (Takeuchi et al. 2011; Yamazaki et al. 2007), and the studies towards the total synthesis, stereochemistry and structure activity of this compound during the last years (Poulsen 2011; Sang et al. 2014; Oku et al. 2014; Oku et al. 2015; Sang et al. 2016; Tsakos et al. 2016) showed the high potency of this compound for medical research.

4.3.2. Aloesaponarin II, 5-hydroxyaloesaponarin II – known compounds – new bioactivity – new source: marine actinobacteria

During the screening of the strain Guam 928 selective growth inhibitions of the Gram-positive test organisms *Staphylococcus aureus* Newman and *Enterococcus faecium* (DSM 20477) including the MRSA and VREF strain were detected. Further analyses showed that strain Guam 928 was the producer of two interesting compounds which were finally identified as aloesaponarin II and its derivative 5-hydroxyaloesaponarin II (3,5,8-trihydroxyaloesaponarin). However, only 5-hydroxyaloesaponarin II could be correlated to the bioactivity against the Gram-positive test organisms. To date this compound was only described to be produced as blue pigment which was isolated together with aloesaponarin II from an actinorhodin-negative mutant of *Streptomyces coelicolor* A3 (2) (Bystrykh et al. 1997). Aloesaponarin II was originally isolated from the higher plant *Aloe saponaria* Haw. I (Yagi et al. 1974). Afterwards, it was found to be produced by a *Streptomyces* mutant of *S. coelicolor* which normally did not produce aloesaponarin II (Bartel at al. 1990; Bystrykh et al 1997) as well as by terrestrial and marine *Streptomyces* sp. strains (Fotso et al. 2003; Cui et al. 2006, Cui et al. 2008).

Finally, the first isolation of 5-hydroxyaloesaponarin II from a bacterial wild type strain was described, in this case the marine *Micromonospora* strain Guam 928. Additionally the specific bioactivity against Gram-positive bacteria including an MRSA and VREF strain was revealed.

4.3.3. Staurosporine – known compounds – new source: marine actinobacteria

In this study, staurosporine was isolated from the marine *Streptomyces* strain ICN21 from rhizosphere sediment of a mangrove plant (*Rhizophora mucronata*) from the coast of Tamil Nadu, India. During the screening process a high bioactivity against the fungal test strains *C. albicans* (DSM 1665) and *M. hiemalis* (DSM 2656) was detected. The following LC-MS analysis of the crude extract identified the active compound as the alkaloid staurosporine.

Staurosporine (AM-2282) was originally isolated from the soil bacterium *Streptomyces staurosporeus* (former known as *Streptomyces* sp. AM-2282) during a search for new alkaloids produced by members from the genus Actinomycetales (Omura et al. 1977). Omura et al. also described antimicrobial activities against fungi and yeast. To date this compound is known to have a strong inhibitory effect against HeLa S3 cells (Tamaoki et al. 1986), platelet aggregation (induced by collagen and ATP) (Oka et al. 1986) and several protein kinases (Meggio et al. 1995) as well as antibacterial and immunosuppressive activities (Gani et al. 2010; Park et al. 2013). Because of the non-selectivity of this compound and the resulting cross-reactivities with other kinases, staurosporine cannot be

used as therapeutic agent (Karaman et al. 2008). However, to use this compound for further medical approaches, many scientists worked on the isolation of staurosporine analogues from different microbial sources or tried to create new derivatives by chemical synthesis to specify the compounds for a particular protein kinase (Park et al. 2013). Finally, most of these analogues were detected as cytotoxic on cellular level or as inhibitor of different protein kinases, but also immunomodulatory as well as neurite outgrowth inhibition activities were found (Park et al. 2013).

5. Conclusion

In this study the strains JS360 ("*Streptomyces cinnabaragriseus*") and *Streptomyces davawensis* were described as separate individual species, 12 *Myxococcus* sp. strains from Wadden Sea sediments as well as four *Streptomyce*s strains from deep sea sediments and sponges from 1092 m depth (North Atlantic Ocean) were isolated, of which strain ASO4 wet could be described as new species. Additionately, strains ICN19 and ICN21, isolated from rhizosphere sediments from mangroves in India, seemed to belong to the same, hopefully novel species and also most of the actinobacterial strains which were isolated by DGC from sponges in Guam have partly a high potential to be described as new. However, due to the describtion of novel species being very time and money consuming this study focused on the describtion of either very interesting compound producers or strains from extraordinary habitats.

Though the question, whether it is profitable to work on marine actinobacteria and myxobacteria for the discovery of new bioactive substances is hard to answer, this study shows that it is at least feasible. 24 actinobacterial and 12 myxobacterial strains were screened for the production of novel secondary metabolites without any success. All strains were isolated from uncommon marine environments like rhizosphere sediments of a mangrove plant (coast of Tamil Nadu, India), and marine sediments as well as sponges from Guam, the deep sea (1092 m, North Atlantic Ocean) and the Wadden Sea (Germany). Furthermore, several enrichment approaches were tested for the isolation of marine bacteria by trying to simulate the natural marine environment. However, in this study only halotolerant and halophilic but no real marine microorganisms could be found. All strains used or isolated were very closely related to known terrestrial myxobacteria and actinobacteria. Even the new deep sea strain ASO4 wet belongs to the genus *Streptomyces* which is mainly known to be a terrestrial actinobacteria. To find new compounds in the future new bacterial families or genera which are indigenous in the marine habitats have to be isolated. Good examples for the theory "new strain = new metabolite" in the marine habitats was found for both, the myxobacteria as well as for the actinobacteria. As described before, the members of the obligate marine genera *Haliangium* and

Ehygromyxa produced the antifungal agent haliangin and the antibiotic salimabromide, respectively. Meanwhile, the marine actinobacterial genera *Salinispora* and *Marinospora* produced the very interesting proteasome inhibitor salinosporamide A and marinomycin A which has a cytotoxic as well as an antibacterial effect against MRSA and VREF. All of these compounds were described to feature novel scaffolds and modes of action. However, even a closely related structure and mode of action to salinosporamide A was detected for the cinnabaramides. These compounds are produced by the terrestrial *Streptomyces* strain.

Finally, to discover novel bioactive compounds, new marine myxobacteria and actinobacteria have to be isolated. Therefore, the focus should be on new and obligate marine genera from undiscovered marine habitats. Furthermore, new isolation techniques have to be established which mimic the natural environment or techniques like the DGC should be established in diverse approaches. Moreover, screening approaches for the isolation of secondary metabolites should be adapted to natural environments of the strains like co-cultivation, increasing or decreasing of the cultivation temperature and pressure, as well as the addition of different components from the marine environment in the cultivation media. However, many molecular biological studies (including the MMC sequence) as well as this thesis showed, that the marine habitat has a great potential for the isolation of marine myxobacteria and actinobacteria.

6. References

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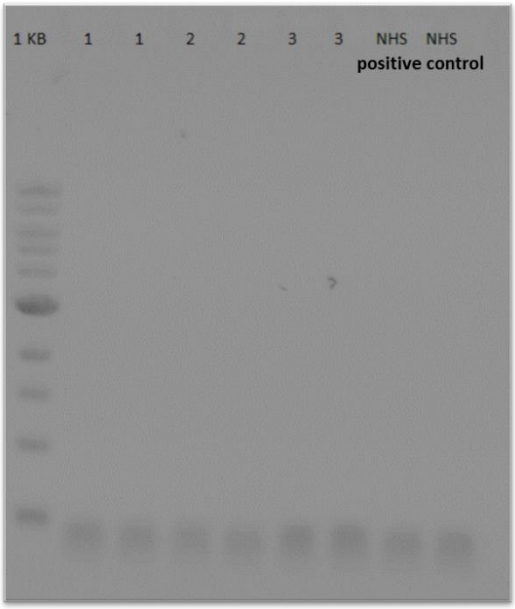
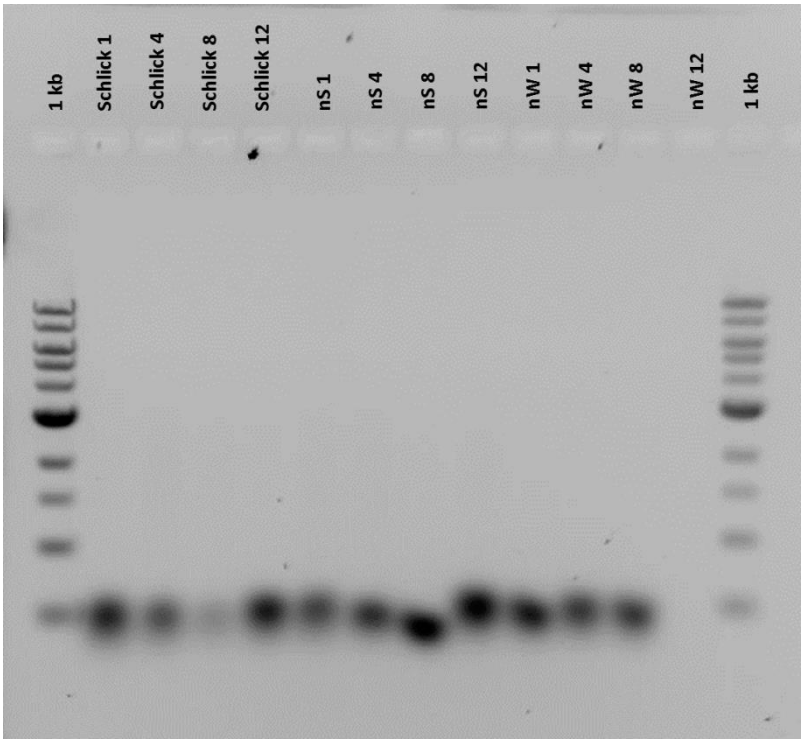
7. Appendix

Detection of MMC sequence in sediment samples (Agarose gels after MMC-PCR)



Agarose gel of the MMC detection PCR for the Janssand samples 1: sediment sample 1; 2: sediment sample 2; 3: rockworm excrutions.



Agarose gel of the MMC detection for the sediment core samples from Neuharlingersiel: Schick 1: mud from 1cm depth; Schick 4: mud from 4 cm depth; Schlick 8: mud from 8cm depth; Schlick 12: mud from 12cm depth; nS1: sediment sample near the beach from 1cm depth; nS4: sediment sample near the beach from 4cm depth; nS8: sediment sample near the beach from 8cm depth; nS12: sediment sample near the beach from 12cm depth; nW1: sediment sample near the water

from 1cm depth; nW4: sediment sample near the water from 4cm depth; nW8: sediment sample near the water from 8cm depth; nW12: sediment sample near the water from 12cm depth.

Closest relatives of the isolated actinobacterial strains based on the 16S rRNA detected via "Blast" search on NCBI.

Closest relatives (type strains) of the Micromonospora strains Guam 928, 1509, 1510, 1582 and 1583 after using the "Blast" tool on NCBI based on the 16S rRNA sequence.

Guam 928 (99%)	
M. aurantiaca	DSM 43813^T
M. halophytica	DSM 43171^T
M. chalcea	DSM 43026^T
M. schwarzwaldiensis	DSM 45708^T
M. echinosora	DSM 43816^T
M. citrea	DSM 43903^T
M.rosaria	DSM 803^T

Guam 1509 (99%)	
M. echinospora	DSM 43816^T
M. citrea	DSM 43903^T
M. aurantiaca	DSM 43813^T
M. sagamiensis subsp. flava	DSM 44887^T
M. endolithica	DSM 44398^T
M. rosaria	DSM 803^T
M. sagamiensis	DSM 43912^T
M. echinofusca	DSM 43913^T
M. chalcea	DSM 43026^T
M. inyonensis	DSM 46123^T
M. chersina	DSM 44151^T
M. pallida	DSM 43817^T

Guam 1510 (99%)	
M. citrea	DSM 43903^T
M. aurantiaca	DSM 43813^T
M. sagamiensis subsp. flava	DSM 44887^T
M. endolithica	DSM 44398^T
M. rosaria	DSM 803^T
M. echinospora	DSM 43816^T
M. echinofusca	DSM 43913^T
M. chersina	DSM 44151^T
M. echinaurantiaca	DSM 43904^T
M. inyonensis	DSM 46123^T
M nigra	DSM 43818^T
M. fulviviridis	DSM 43906^T
M. viridifaciens	DSM 43909^T
M. pallida	DSM 43817^T

Guam 1582 (99%)	
M. echinospora	DSM 43816^T
M. citrea	DSM 43903^T
M. aurantiaca	DSM 43813^T
M. sagamiensis subsp. flava	DSM 44887^T
M. endolithica	DSM 44398^T
M. rosaria	DSM 803^T
M. sagamiensis	DSM 43912^T
M. echinofusca	DSM 43913^T
M. chalcea	DSM 43026^T
M. inyonensis	DSM 46123^T
M. chersina	DSM 44151^T
M. pallida	DSM 43817^T
M. halophytica	DSM 43171^T

Guam 1583 (99%)	
M. aurantiaca	DSM 43813^T
M. sagamiensis subsp. flava	DSM 44887^T
M. endolithica	DSM 44398^T
M. rosaria	DSM 803^T
M. echinospora	DSM 43816^T
M. echinofusca	DSM 43913^T
M. sagamiensis	DSM 43912^T
M. chersina	DSM 44151^T

Closest relatives (type strains) of the Streptomyces strains Guam 1322, 1285 and after using the "Blast" tool on NCBI based on the 16S rRNA sequence.

Guam 1322 (99%)		**Guam 1285** (99%)	
S. tendae	DSM 40101^T	*S. tendae*	DSM 40101^T
S.violaceorubidus	DSM 41478^T	*S.violaceorubidus*	DSM 41478^T
S. djakartensis	DSM 40743^T	*S. djakartensis*	DSM 40743^T
S. collinus	DSM 40129^T	*S. collinus*	DSM 40129^T
S. griseoflavus	DSM 40456^T	*S. griseoflavus*	DSM 40456^T
S. malachitofuscus	DSM 40332^T	*S. malachitofuscus*	DSM 40332^T
S. albogriseolus	DSM 40003^T	*S. paradoxus*	DSM 43350^T
S. tritolerans	DSM 41899^T	*S. albogriseolus*	DSM 40003^T
S. paradoxus	DSM 43350^T	*S. viridochromogenes*	DSM 40110^T
S. viridochromogenes	DSM 40110^T	*S. rubrogriseus*	DSM 41477^T
S. rubrogriseus	DSM 41477^T	*S. lienomycini*	DSM 41475^T
S. lienomycini	DSM 41475^T	*S. griseorubens*	DSM 40160^T
S. marokkonensis	DSM 41918^T	*S. marokkonensis*	DSM 41918^T
S. griseorubens	DSM 40160^T	*S. lienomycini*	DSM 41475^T
S. chattanoogensis	DSM 40002^T	*S. parvulus*	DSM 40048^T
S. parvulus	DSM 40048^T	*S. tuirus*	DSM 40505^T
S. tuirus	DSM 40505^T	*S. chattanoogensis*	DSM 40002^T
S. tricolor	DSM 41704^T	*S. flaveolus*	DSM 40061^T
S. anthocyanicus	DSM 41422^T	*S. geysiriensis*	DSM 40742^T
S. violaceoruber	DSM 40049^T	*S. tricolor*	DSM 41704^T
S. coelescence	DSM 40421^T	*S. anthocyanicus*	DSM 41422^T
S. flaveolus	DSM 40061^T	*S. violaceoruber*	DSM 40049^T
S. geysiriensis	DSM 40742^T	*S. althioticus*	DSM 40092^T
S. althioticus	DSM 40092^T	*S. mutabilis*	DSM 40169^T
S. ambofaciens	DSM 40053^T	*S. ambofaciens*	DSM 40053^T
S. chartreusis	DSM 40085^T	*S. rochei*	DSM 40231^T
S. mutabilis	DSM 40169^T	*S. enissocaesilis*	DSM 41454^T
S. variabilis	DSM 40053^T	*S. glaucescens*	DSM 40155^T
S. labedae	DSM 41446^T	*S. luteogriseus*	DSM 40483^T
S. erythrogriseus	DSM 40116^T	*S. violaceolatus*	DSM 40438^T
S. violaceolatus	DSM 40438^T	*S. vinaceusdrappus*	DSM 40470^T
S. griseoinicanatus	DSM 40274^T	*S. plicatus*	DSM 40319^T
S. humiferus	DSM 43030^T	*S. matensis*	DSM 40188^T
S. rochei	DSM 40231^T	*S. ambofaciens*	DSM 40053^T
S. enissocaesilis	DSM 41454^T	*S. humiferus*	DSM 43030^T
S. glaucescens	DSM 40155^T	*S. violaceolatus*	DSM 40438^T
S. luteogriseus	DSM 40483^T	*S. tuirus*	DSM 40505^T
S. vinaceusdrappus	DSM 40470^T	*S. almquistii*	DSM 40447^T
S. plicatus	DSM 40319^T	*S. variabilis*	DSM 40053^T
S. matensis	DSM 40188^T	*S. labedae*	DSM 41446^T
S. almquistii	DSM 40447^T	*S. lomondensis*	DSM 41428^T
S. lomondensis	DSM 41428^T	*S. erythrogriseus*	DSM 40116^T
S. misionensis	DSM 40306^T	*S. griseoincarnatus*	DSM 40274^T

S. violaceochromogenes	DSM 40181^T	*S. chartreusis*	DSM 40085^T
S. viridis	DSM 40381^T	*S. viridis*	DSM 40381^T
S. malachitospinus	DSM 41828^T	*S. malachitospinus*	DSM 41828^T
S. iakyrus	DSM 40482^T	*S. iakyrus*	DSM 40482^T
S. purpurascens	DSM 40310^T	*S. purpurascens*	DSM 40310^T
S. flavoviridis	DSM 40153^T	*S. flavoviridis*	DSM 40153^T
S. pilosus	DSM 40097^T	*S. pilosus*	DSM 40097^T
S. eurythermus	DSM 40014^T	*S. misionensis*	DSM 40306^T
S. levis	DSM 41458^T	*S. arenae*	DSM 40293^T
S. nogalater	DSM 40546^T	*S. violaceus*	DSM 40082^T
S. violaceus	DSM 40082^T	*S. roseoviolaceus*	DSM 40277^T
S. roseoviolaceus	DSM 40277^T	*S. eurythermus*	DSM 40014^T
S. phaeoluteichromatogenes	DSM 41898^T	*S. levis*	DSM 41458^T
S. olivaceus	DSM 40072^T	*S. olivaceus*	DSM 40072^T
S. pactum	DSM 40530^T	*S. bellus*	DSM 40185^T
S. coerulescens	DSM 40146^T	*S. azureus*	DSM 40106^T
S. bellus	DSM 40185^T	*S. pactum*	DSM 40530^T
S. azureus	DSM 40106^T	*S. althioticus*	DSM 40092^T
S. arenae	DSM 40293^T	*S. coerulescens*	DSM 40146^T
		S. nogalater	DSM 40546^T

Closest relatives (type strains) of the *Streptomyces* strains A-, B- and C SedH 10^{-3} after using the "Blast" tool on NCBI based on the 16S rRNA sequence.

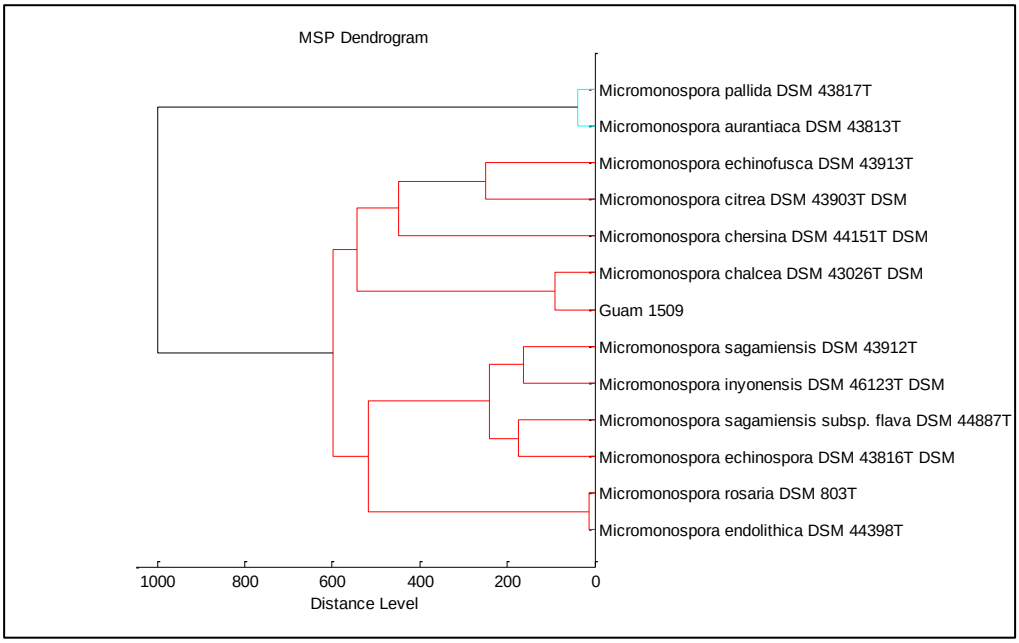
ASedH 10^{-3} (99%)		**BSedH 10^{-3}** (99%)		**CSedH 10^{-3}** (99%)	
S. anulatus	DSM 40361^T	*S. anulatus*	DSM 40361^T	*S. anulatus*	DSM 40361^T
S. caviscabis	ATCC 51928^T	*S. caviscabis*	ATCC 51928^T	*S. rubiginosohelvolus*	DSM 40176^T
S. fulvissimus	DSM 40593^T	*S. fulvissimus*	DSM 40593^T	*S.pluricoloresces*	DSM 40019^T
S. rubiginosohelvolus	DSM 40176^T	*S. rubiginosohelvolus*	DSM 40176^T	*S. globisporus*	DSM 40199^T
S. microflavus	DSM 40331^T	*S. microflavus*	DSM 40331^T	*S. caviscabies*	ATCC 51928^T
S. cyaneofuscatus	DSM 40148^T	*S. cyaneofuscatus*	DSM 40148^T	*S. fulvissimus*	DSM 40593^T
S. griseus	DSM 40236^T	*S. griseus*	DSM 40236^T	*S. microflavus*	DSM 40331^T
S. mediolani	DSM 41647^T	*S. mediolani*	DSM 41647^T	*S. cyaneofuscatus*	DSM 40148^T
S. parvus	DSM 40348^T	*S. parvus*	DSM 40348^T	*S. griseus*	DSM 40236^T
S. argenteolus	DSM 40226^T	*S. argenteolus*	DSM 40226^T	*S. argenteolus*	DSM 40226^T
S. badius	DSM 40139^T	*S. badius*	DSM 40139^T	*S. mediolani*	DSM 41647^T
S. sindenensis	DSM 40255^T	*S. sindenensis*	DSM 40255^T	*S. badius*	DSM 40139^T
S. fulvorobeus	DSM 41455^T	*S. fulvorobeus*	DSM 41455^T	*S. fulvorobeus*	DSM 41455^T
S. globisporus	DSM 40199^T	*S. globisporus*	DSM 40199^T	*S. cinereorectus*	DSM 41469^T
S. baarnensis	DSM 40232^T	*S. baarnensis*	DSM 40232^T	*S. microflavus*	DSM 40331^T
S. cinereorectus	DSM 41469^T	*S. cinereorectus*	DSM 41469^T	*S. baarnensis*	DSM 40232^T
S. pluricolorescens	DSM 40019^T	*S. pluricolorescens*	DSM 40019^T	*S. parvus*	DSM 40348^T
S. griseolus	DSM 40067^T	*S. griseolus*	DSM 40067^T	*S. flavovirens*	DSM 40062^T
S. halstedii	DSM 40068^T	*S. halstedii*	DSM 40068^T	*S. griseolus*	DSM 40067^T
S. flavovirens	DSM 40062^T	*S. flavovirens*	DSM 40062^T	*S. puniceus*	DSM 40083^T
S. tanashiensis	DSM 40195^T	*S. tanashiensis*	DSM 40195^T	*S. halstedii*	DSM 40068^T
S. pulveraceus	DSM 41657^T	*S. pulveraceus*	DSM 41657^T	*S. cyaneus*	DSM 40108^T

S. bacillaris	DSM 40598^T	*S. bacillaris*	DSM 40598^T	*S. tanashiensis*	DSM 40195^T
S. finayi	DSM 40218^T	*S. finayi*	DSM 40218^T	*S. pulveraceus*	DSM 41657^T
S. atroolivaceus	DSM 40137^T	*S. atroolivaceus*	DSM 40137^T	*S. finlayi*	DSM 40218^T
S. clavifer	DSM 40843^T	*S. clavifer*	DSM 40843^T	*S. atroolivaceus*	DSM 40137^T
S. nitrosporeus	DSM 40023^T	*S. nitrosporeus*	DSM 40023^T	*S. nitrosporeus*	DSM 40023^T
S. mutomycini	DSM 41691^T	*S. mutomycini*	DSM 41691^T	*S. clavifer*	DSM 40843^T
S. albolongus	DSM 40570^T	*S. albolongus*	DSM 40570^T	*S. bacillaris*	DSM 40598^T
S. cavourensis	DSM 40300^T	*S. cavourensis*	DSM 40300^T	*S. mutomycini*	DSM 41691^T
S. atratus	DSM 41673^T	*S. atratus*	DSM 41673^T	*S. sanglieri*	DSM 41791^T
S. gelaticus	DSM 40065^T	*S. gelaticus*	DSM 40065^T	*S. atratus*	DSM 41673^T
S. spiroverticillatus	DSM 40036^T	*S. spiroverticillatus*	DSM 40036^T	*S. gelaticus*	DSM 40065^T
				S. spiroverticillatus	DSM 40036^T

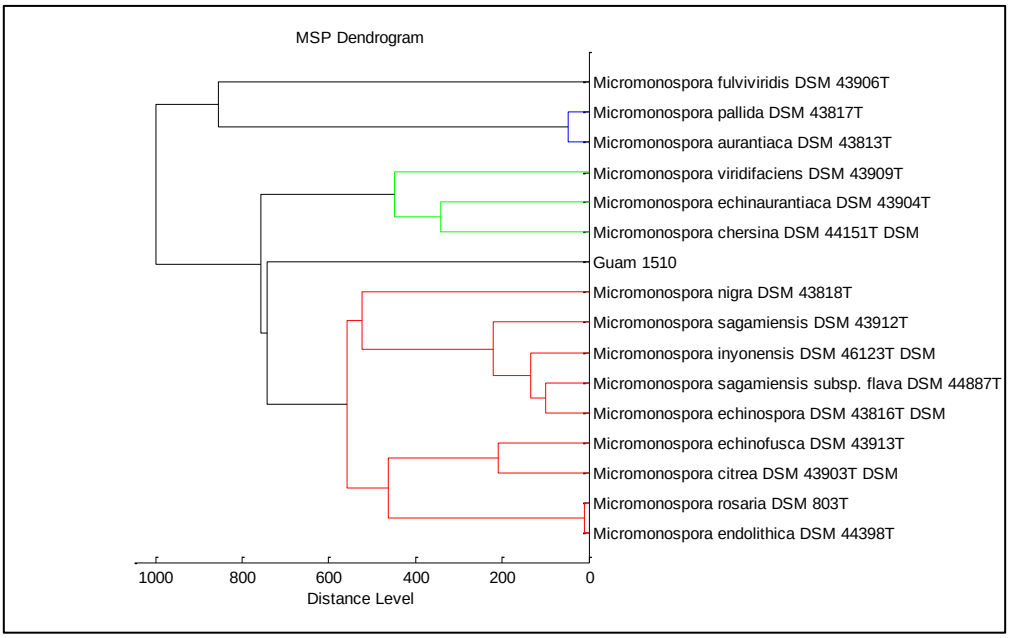
Closest relatives (type strains) of the Streptomyces S. davawensis, S. cinnabarinus and Streptomyces strain JS360 (*S. cinnabaragriseus*) after phylogenetic analysis of the 16S rRNA serquence of the Prof. Dr. Peter Kämpfer and his team (University of Gießen).

Strain name	**Strain no.**
Streptomyces davawensis	JCM 4913 = DSM 101723^T
Streptomyces cinnabarinus	DSM 40467^T
Streptomyces cinnabaragriseus	JS360 = DSM 101724^T
Streptomyces avermitilis	DSM 46492^T
Streptomyces flavovariabilis	DSM 41479^T
Streptomyces novaecaesareae	DSM 40358^T
Streptomyces alboniger	DSM 40043^T
Streptomyces cellostaticus	DSM 40189^T
Streptomyces bobili	DSM 40056^T
Streptomyces galilaeus	DSM 40481^T
Streptomyces griseochromogenes	DSM 40499^T
Streptomyces pseudovenezuelae	DSM 40212^T
Streptomyces phaeoluteigriseus	DSM 41896^T
Streptomyces atriruber	DSM 41860^T
Streptomyces resistomycificus	DSM 40133^T
Streptomyces yokosukanensis	DSM 40224^T
Streptomyces olivochromogenes	DSM 40451^T
Streptomyces corchorusii	DSM 40340^T
Streptomyces longwoodensis	DSM 41677^T
Streptomyces curacoi	DSM 40107^T
Streptomyces antibioticus	DSM 40234^T
Streptomyces canus	DSM 40017^T
Streptomyces ciscaucasicus	DSM 40275^T
Streptomyces griseorubiginosus	DSM 40469^T
Streptomyces phaeopurpureus	DSM 40125^T
Streptomyces griseruber	DSM 40281^T

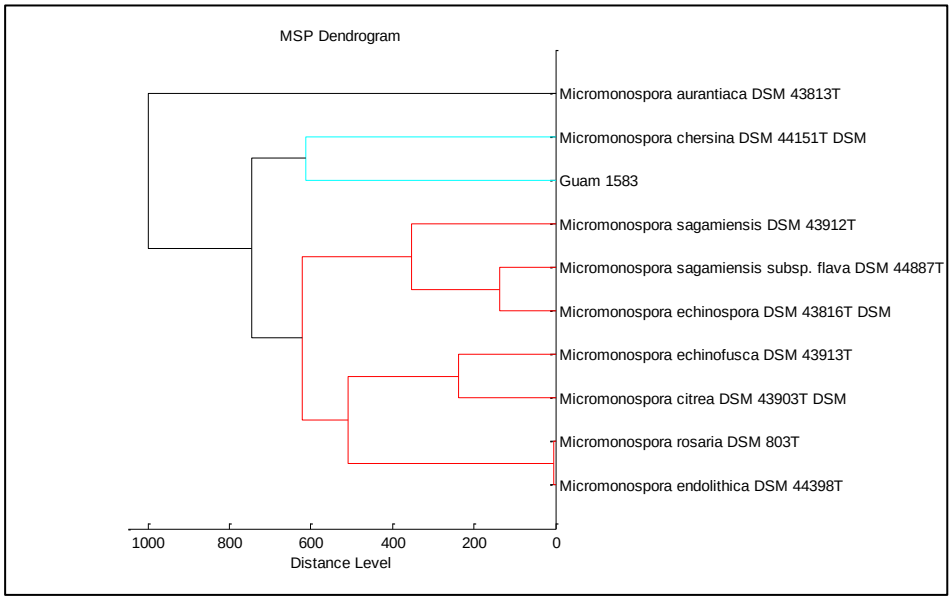
MALDI TOF dendrogrammes of Guam strains



MALDI-TOF dendrogram of the *Micomonospora* strain Guam 1509 and its closest relatives (type strains).



MALDI-TOF dendrogram of the *Micromonospora* strain Guam 1510 and its closest relatives (type strains).



MALDI-TOF dendrogram of the *Micromonospora* strain Guam 1583 and its closest relatives (type strains).

MIC of extracts from isolated Actinobacteria from Guam and India on the standard test panel.

MIC of the extracts from the isolated actinobacteria from sponges and marine sediment from Guam against the standard test panel in different media: [4] 5254 medium; [5] 5254 sea water medium; [6] 5294 medium; [7] 5294 sea water medium.

	Fungi			**Gram +**				**Gram -**			
	P. anomala (DSM 6766)	*C. albicans* (DSM 1665)	*M. hiemalis* (DSM 2656)	*M. luteus* (DSM 20030)	*B. subtilis* (DSM 10)	*S. aureus* Newman*	*M. phlei* (DSM 43070)	*C. violaceum* (DSM 30191)	*P. aeruginosa* (DSM 50071)	*E.coli* (DSM 1116)	*E.coli* TolC
Guam 928[4]	-	-	-	-	E	-	-	A	-	-	-
Guam 928[5]	-	-	-	H	H	E	B	A	-	-	B
Guam 928[6]	-	-	-	-	F	-	-	-	-	-	-
Guam 928[7]	-	-	-	F	E	D	-	-	-	-	-
Guam 1257[4]	-	-	-	A	A	-	-	-	-	-	-
Guam 1257[5]	-	-	-	C	B	A	B	A	-	A	-
Guam 1257[6]											
Guam 1257[7]											
Guam 1285[4]	-	-	-	H	H	H	B	F	A	C	A
Guam 1285[5]	-	-	-	H	H	H	B	E	A	C	A
Guam 1285[6]											

Guam 1285[7]											
Guam 1322[4]	-	-	-	H	H	H	B	D	-	-	-
Guam 1322[5]	-	-	-	H	H	H	B	D	-	B	B
Guam 1322[6]											
Guam 1322[7]											
Guam 1509[4]	-	-	-	A	B	B	-	A	-	-	-
Guam 1509[5]	-	-	-	B	B	-	-	-	-	-	-
Guam 1509[6]	-	-	A	C	D	C	-	A	-	-	-
Guam 1509[7]	-	-	-	B	C	B	-	-	-	-	-
Guam 1510[4]	-	-	-	B	D	A	B	A	-	-	-
Guam 1510[5]	-	-	-	C	C	A	B	-	-	-	-
Guam 1510[6]											
Guam 1510[7]											
Guam 1566[4]	-	-	-	C	G	B	-	-	-	-	-
Guam 1566[5]	-	-	-	-	G	-	-	-	-	-	-
Guam 1566[6]	-	-	-	F	G	E	-	-	-	-	-
Guam 1566[7]	-	-	-	C	B	-	-	A	-	-	A
Guam 1582[4]	-	-	-	F	-	-	-	-	-	-	-
Guam 1582[5]	-	A	-	G	H	D	-	-	-	-	-
Guam 1582[6]	-	-	A	D	E	B	-	A	A	-	D
Guam 1582[7]	-	-	A	G	G	D	-	-	-	-	-
Guam 1583[4]	-	-	A	E	D	A	-	-	-	-	-
Guam 1583[5]	-	-	A	G	G	E	-	-	-	-	-
Guam 1583[6]	-	-	A	D	D	B	-	A	-	-	C
Guam 1583[7]	-	-	A	F	F	D	-	-	-	-	-

MIC of the extracts from the isolated actinobacteria from rhizosphere sediment from mangroves from India against the standard test panel in different media: [4] 5254 medium; [5] 5254 sea water medium; [6] 5294 medium; [7] 5294 sea water medium.

	Fungi			**Gram +**				**Gram -**			
	P. anomala (DSM 6766)	*C. albicans* (DSM 1665)	*M. hiemalis* (DSM 2656)	*M. luteus M. luteus* (DSM 20030)	*B. subtilis* (DSM 10)	*S. aureus* Newman*	*M. phlei* (DSM 43070)	*C. violaceum* (DSM 30191)	*P. aeruginosa* (DSM 50071)	*E.coli* (DSM 1116)	*E.coli* TolC
ICN4[4]	-	-	-	G	H	G	-	B	-	-	B
ICN4[5]	-	-	-	G	H	G	-	C	-	-	D
ICN4[6]											
ICN4[7]											
ICN16[4]	-	-	A	-	B	-	-	-	-	-	-
ICN16[5]	-	-	B	F	G	E	A	-	-	-	A
ICN16[6]	-	-	A	A	A	A	-	-	-	-	-
ICN16[7]	-	-	-	-	-	-	-	-	-	-	-
ICN18[4]	-	-	B	H	H	H	B	D	-	A	B
ICN18[5]	-	-	A	F	H	F	-	C	-	-	-
ICN18[6]	C	C	D	H	H	H	G	H	B	G	H
ICN18[7]	-	-	-	F	F	F	-	B	-	-	A
ICN19[4]	C	H	H	H	H	H	H	D	-	-	F
ICN19[5]	C	F	F	H	H	D	F	-	-	-	C
ICN19[6]	F	F	G	H	H	H	H	F	-	A	H
ICN19[7]	D	E	E	H	G	D	E	-	-	-	C
ICN21[4]	C/F*	H	H	A	C	B	B	-	-	-	C
ICN21[5]	B/E*	F	F	B	D	A	B	-	-	-	C
ICN21[6]	F	H	H	B	C	-	A	-	-	-	E
ICN21[7]	D	F	F	A	C	-	-	A	-	-	-
ICN26[4]	-	-	A	C	E	D	A	A	-	-	-
ICN26[5]	-	-	-	-	-	-	-	-	-	-	-
ICN26[6]	-	-	-	C	A	C	-	A	-	-	-
ICN26[7]	F	H	H	A	B	-	B	A	-	-	E
ICN28[4]	-	-	A	D	E	C	-	-	-	-	-

ICN28[5]	E	E	D	C	E	-	B	-	-	-	-
ICN28[6]	D	D	D	H	H	H	A	B	-	-	-
ICN28[7]	B/C*	B	B	D	E	C	-	-	-	-	-

Sequences of bacterial isolates

Guam 928

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Guam 1257

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Guam 1285

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Guam 1322

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Guam 1509

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Guam 1510

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Guam 1566

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Guam 1582

TGCAAGTCGAGCGGAAGGCCCTTCGGGGTACTCGAGCGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCC
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Guam 1583

GACGGCTCCCTCCACAAGGGTTGGGCCACCGGCTTCGGGTGTTGCCGACTTTCGTGACGTGACGGGCGGTGT
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GCCGCCAGCGTTCGTCCTGAGCCA

ASO4 wet

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A-Sed H10^{-3}

GCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACTCTGTCCCGCATGGGACGGGG
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B-Sed H10^{-3}

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CGA

C-Sed H10^{-3}

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ICN16

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ICN18

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ICN19

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ICN21

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ICN26

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ICN27

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Streptomyces davawensis

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AGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
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CGGGGCTTAACCCCGAGTCTGCATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGT
GTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGC
TGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGAACT
AGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCG
CAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAAC
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TCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGG
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CTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGTCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCC
ATGAAGTTGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAA
GGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCT

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CGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACG
GGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATC
ACTCTCGCAGGCATCTGTGAGGGTCGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTG
GTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCG
ACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTAC
CTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAA
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CATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGAT
ATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGG
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TTGACATACACCGGAAACGTCCAGAGATGGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCT
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GCGAATCTCAAAAAGTCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGGAGTTGCTAGT
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CGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGG
ACGAAGTCGTAACAAGGTAGCCGTACCGGAAGG

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GCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT
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GCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
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GGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCT
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WS 1.1

CTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAGC
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CCGGATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGAT
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GACGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCA
ATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGTGTGATGAAGGTCTTTGGATTGTAAAGCACTTTCGACCG
GGAAGAAAACCCGTTGGCTAACATCCAACGGCTTGACGGTACCGGGAGAAGAAGCACCGGCTAACTCTGTGC
CAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGCGCGTGTAGGCGGCG
TGACAAGTCGGGTGTGAAAGCCCTCAGCTCAACTGAGGAAGTGCGCCCGAAACTGTCGTGCTTGAGTGCCGG
AGAGGGTGGCGGAATTCCCCAAGTAGAGGTGAAATTCGTAGATATGGGGAGGAACACCGGTGGCGAAGGCG
GCCACCTGGACGGTAACTGACG

WS 2.1

TAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAGCGGCGCACGGGTGCGTAACACGTGGAT
AATCTGCCTGGATGCTCGGGATAACCAGTCGAAAGATTGGCTAATACCGGATAAGCCCACGGTTTCTTCGGAG
ACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGATGAGTCCGCGGCCCATCAGCTAGTTGGC
GGGGTAATGGCCCACCAAGGCAACGACGGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAGCCTGACGCAGCAACG
CCGCGTGTGTGATGAAGGTCTTCGGATTGTAAAGCACTTTCGACCGGGACGAAAACCCGTAGCCCAACACGCT
ACGGCT

WS 2.3

TGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAGCGGCGCACGGGTGC
GTAACACGTGGATAATCTGCCTGGATGCTCGGGATAACCAGTCGAAAGATTGGCTAATACCGGATAAGCCCAC
GGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGATGAGTCCGCGGCCCA
TCAGCTAGTTGGCGGGGTAATGGCCCACCAAGGCAACGACGGGTAGCTGGTCTGAGAGGACGATCAGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAGCCT
GACGCAGCAACGCCGCGTGTGTGATGAAGGTCTTCGGATTGTAAAGCACTTTCGACCGGGACGAAAACCCGT
AGCCCAACACGCTACGGCTtGACGGTACCGGGAGAAGAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTA
ATACA

WS 3.3

GGCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGA
GCGGCGCACGGGTGCGTAACACGTGGATAATCTGCCTGGATGCTCGGGATAACCAGTCGAAAGATTGGCTAA
TACCGGATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAG
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AGGACGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCG
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GAGAGGGTGGCGGAATTCCCCAAGTAGAGGTGAAATTCGTAGATATGGGGAGGAACACCGGTGGCGAAGGC
GGCCACCTGGACGGTAACTGACGCTGAGAC

WS 3.4

ATAGGGGCAACCCTTAGTAGAGCGGCGCACGGGTGCGTAACACGTGGATAATCTGCCTGGATGCTCGGGATA
ACCAKACGAAAGATTGGCTAATACCGGATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCT
GTATACAAGCTATCACAACCAGATGAGTCCGCGGCCCATCAGCTAGTTGGCGGGGTAATGGCCCACCAAGGC
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CGGATTGTAAAGCACTTTCGACCGGGACGAAAACCCGTAGCCCAACACGCTACGGCTTGACGGTACCGGGAG
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CGAAACTGTCGTGCTTGAGTGCCGGAGAGGGTGGCGGAATTCCCCAAGTAGAGGTGAAATTCGTAGATATGG
GGAGGAACACCGGTGGCGAAGGCGGCCACCTGGACGGTAACTGACGCTGAGACGCGAAAGCGTGGGGAGC
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WS 4.1

GCTCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAG
CGGCGCACGGGTGCGTAACACGTGGATAATCTGCCTGAGTGCTCGGGATAACCAGTCGAAAGATTGGCTAAT
ACCGGATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACATTCAGA
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GGACGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGC
AATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGTGTGATGAAGGTCTTTGGATTGTAAAGCACTTTCGACC
GGGAAGAAAACCCGTTGGCTAACATCCAACGGCTtGACGGTACCGGGAGAAGAAGCACCGGCTAACTCTGTG
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GTGACAAGTCGGGTGTGAAAGCCCTCAGCTCAACTGAGGAAGTGCGCCCGAAACTGTCGTGCTTGANGTGCC
GGAGAGGGTGGCGGAATTCCCCAAGTAGAGGTGAAATTCGTAGATATGGGGAGGAACACCGGTGGCGAAGG
CGGCCACCTGGACGGTAACTGACGCTGAGACG

WS 5.1

CGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAGCGGCGCAC
GGGTGCGTAACACGTGGATAATCTGCCTGAGTGCTCGGGATAACCAGTCGAAAGATTGGCTAATACCGGATA
AGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACATTCAGATGAGTCCG
CGGCCCATCAGCTAGTTGGCGGGGTAATGGCCCACCAAGGCAACGACGGGTAGCTGGTCTGAGAGGACGATC
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCG
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CGCGGTAATACAGAGGGTGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGCGCGTGTAGGCGGCGTGACAAG
TCGGGTGTGAAAGCCCTCAGCTCAACTGAGGAAGTGCGCCCGAAACTGTCGTGCTTGAGTGCCGGAGAGGGT
GGCGGAATTC

WS 9.1

TCAGAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAGCG
GCGCACGGGTGCGTAACACGTGGATAATCTGCCTGGATGCTCGGGATAACCAGTCGAAAGATTGGCTAATAC
CGGATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGATG
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GACAAGTCGGGTGTGAAAGCCCTCAGCTCAACTGAGGAAGTGCGCCCGAAACTGTCGTGCTTGAGTGCCGGA
GAGGGTGGC

WS 15.1

GAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAGCGGC
GCACGGGTGCGTAACACGTGGATAATCTGCCTGGATGCTCGGGATAACCAGTCGAAAGATTGGCTAATACCG
GATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGATGAG
TCCGCGGCCCATCAGCTAGTTGGCGGGGTAATGGCCCACCAAGGCAACGACGGGTAGCTGGTCTGAGAGGAC
GATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATG
GGCGAAAGCCTGACGCAGCAACGCCGCGTGTGTGATGAAGGTCTTCGGATTGTAAAGCACTTTCGACCGGGA
CGAAAACCCGTAGCCCAACACGCTACGGCTTGACGGTACCGGGAGAAGAAGCACCGGCTAACTCTGTGCCAG
CA

WS 21.3

GAACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAGCGCGAATAGGGGCAACCCTTAGTAGAGCGGC
GCACGGGTGCGTAACACGTGGATAATCTGCCTGAGTGCTCGGGATAACCAGTCGAAAGATTGGCTAATACCG
GATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACATTCAGATGAG
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ACCTGGACGGTAACTGACG

WS 28.1

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CGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAAT
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AGGGTGGCGGAATTCCCCAAGTAGAGGTGAAATTCGTAGATATGGGGAGGAACACCGGTGGCGAAGGCGGC
CACCTGGACGGTAACTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGAACTAGGTGTCGTGGGAGTTGACCCCCGCGGTGCCGTAG

WS 28.3

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GATAAGCCCACGGTTTCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGATGAG
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GATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATG
GGCGAAAGCCTGACGCAGCAACGCCGCGTGTGTGATGAAGGTCTTCGGATTGTAAAGCACTTTCGACCGGGA
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AAGTCGGGTGTGAAAGCCCTCAGCTCAACTGAGGAAGTGCGCCCGAAACTGTCGTGCTTGAGTGCCGGAGAG
GGTGGCGGAATTCCCCAAGTAGAGGTGAAATTCGTAGATATGGGGAGGAACACCGGTGGCGAAGGCGGCCA
CCTGGACGGTAACTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGAACTAGGTGTCGTGGGAGTTGACCCCC

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